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Measurement of Horticulture Produce Quality

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Abstract

The aim of this work was to develop amperometric biosensors, for the detection of key analytes that affect horticulture produce quality.

Information was gathered relating to the interaction between key analytes, such as amino acids, organic acids and sugars and their effect on produce quality. Trends of these key analytes changed in the produce over time, thus their measurement can indicate ripeness. A taste panel analysis on six varieties of tomatoes was therefore carried out. The key analytes were then measured by conventional means and the data was subjected to Principal Component Analysis (PCA). The results indicate that total organic acids correlate well with perceived sweetness of tomatoes.

Individual amperometric biosensors for the measurement of L-malic acid, D-glucose, L-ascorbic acid, L-amino acids, and L-glutamic acid were developed during the course of this study. These individual biosensors were tested with real samples and the results were compared with standard photometric tests. The biosensors generally gave high precision values. The L-malic acid biosensor showed a high correlation against the standard method (accuracy = > 87.3 % when testing real samples). One of the problems encountered when testing real samples was the effect of interferent species. Therefore, quantification of the interferent effect was achieved by testing the sample with and without enzyme immobilised onto the working electrode. By calculating the difference in current, an accurate concentration of the target analyte could be measured.

Finally, it was understood through the course of this work that measurement of only single analytes is insufficient in determining the quality of horticulture produce. Therefore a multianalyte biosensor was constructed. D-glucose, L-malic acid, and L-ascorbic acid, were tested simultaneously using this biosensor array. The average accuracy of this method, as compared to detection of single analytes was 90 %.

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List of Publications

Setford, S.J., Arif, M, Shields, J, Burton, K.S., Tothill, I.E. (2000). Enzyme based biosensor array for evaluation of horticulture produce quality. *Biosensors 2000*, San Diego, USA, 24-26 May.

Arif, M, Setford, S.J., Tothill, I.E., Shields, J, Burton, K.S. (2000). A mediatorless biosensor for the detection of malic acid in horticultural produce. *Biosensors 2000*, San Diego, USA, 24-26 May.

Arif, M, Setford, S.J., Tothill, I.E., Shields, J, Burton, K.S. (2000). Measurement of Horticulture Quality. *Presented at the First Postgraduate Research Conference, Cranfield University,Silsoe, UK, 28-29TH June 2000*, A-38, 21.

Setford, S.J., Arif, M, Shields, J, Burton, K.S., Tothill, I.E. (2000). Evaluation of horticultural produces quality using a biosensor array. *Agricultural Engineering into the third Millennium, International Conference AGEng 2000*, Warwick, UK, 2-7 July.

Arif, M, Setford, S.J., Burton, K.S., Tothill, I.E. (2002). L-malic acid biosensor for field-based evaluation of apple, potato and tomato horticulture produce. *Analyst*. 127, 104-108.

Arif, M, Giakoumaki, E, Setford, S.J., Tothill, I.E. (2002). Development of a sensor array for food diagnosis. *Biosensors 2002*, Kyoto, Japan, 15-17 May.

List of Awards related directly to this work

- Paper 4 was awarded the Douglas Bomford Trust Award for best paper at the AgEng 2000, Warwick
- Awarded the AJ Banks Trust award for 2002 from Society of Chemistry and Industry.
- Winner of Society of Experimental Biology (SOB) Travel award (2002) – for travel to Biosensors 2002, Kyoto, Japan.
- Winner of the Royal Society of Chemistry travel award (2002) - For travel to Biosensors 2002, Kyoto, Japan.

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Commonly Used Abbreviations

Abs.	Absorbance
AAO	Amino Acid oxidase
ADP	Adenosine diphosphate
Ag/AgCl	Silver/Silver Chloride
CA	Cellulose acetate
CV	Coefficient of Variation
GOD	Glucose oxidase
GD	Glutamate dehydrogenase
HEC	hydroxyethylcellulose
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
HRI	Horticulture Research International
HRP	Horseradish Peroxidase
LOD	Limit of Detection
MDH	Malate dehydrogenase
ME	Malic enzyme
M_r	Relative Molecular Mass
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
OD	Optical Density Units
P	Phosphate
RO	Reverse Osmosis
SD	Standard Deviation
SPE	Screen Printed Electrode
UV	Ultraviolet

1 INTRODUCTION

A healthy human diet is dependent upon the availability of high quality nutrition foodstuffs at realistic prices. This has led to a demand from consumers for a competitive food industry that is ever striving to improve its produce and at the same time to minimise its costs in order to ensure profitability in a globally competitive market. Due to the expansion of the horticulture industry, the consumer has been given a wider choice of product, and therefore it is in the interests of the manufacturer to provide food of the highest quality. In order to fulfil consumer demands the manufacturer must cater to consumer taste.

The research presented here focuses on developing electrochemical (amperometric) biosensors to measure produce quality within the low margin, high volume food industry. It has the specific aim of creating biosensors that are simple and thus user friendly, and low cost, being attractive to the food industry. This research is carried out as part of DEFRA (Department for Environment, Food and Rural Affairs) project. This chapter will address the following.

- *To give the problem statement and motivation of this research.*
- *To give the aims of this thesis*
- *To present the thesis layout.*

1.1 Problem Statement and Motivation

The problem statement of this research is as follows.

Development of a rapid detection system to measure Horticulture Quality.

The consumers' choice for a particular type of food is usually judged by the consumers' sensory characteristics. This includes the texture of food, which is determined by the moisture and fat contents, as well as the amounts of carbohydrates and proteins (Szczesniak 1963). The foods taste, which can also be attributed to the saltiness, sweetness, bitterness and acidity, could also be a factor for consumer choice. Other factors include the flavour, aroma shape and colour of the food (Fellows 1996). Simple tests like measuring the wet and dry weights of food produce are used in industry but these tests cannot give a full understanding of consumer preferences. More complex tests such as liquid chromatography, gas chromatography and spectral techniques are able to explain the internal characteristics of the food sample, but are expensive to use and also trained laboratory staff are needed to run the experiments. The time needed to perform these experiments could also be crucial to the manufacturer. The need therefore arises to develop a method that is able to measure the internal characteristics of food e.g. measurement of analytes such as glucose which can measure the sweetness of the fruit, or L-malic acid which could indicate how sour the fruit is, and also show if the fruit has ripened. This method must however not contain the disadvantages of the above techniques. Biosensors are useful measuring tools that can play a prominent

part in food processing and quality control, because of their compactness in size, portability and specificity for a given analyte.

1.2 Research Aim

The aim of this research is to explore the field of biosensors, for developing techniques that are capable of dealing with the challenges posed by three features of the horticulture industry, design optimisation problems: multiple objectives, constraints and interaction with the environment, i.e. biosensor must be designed for simplicity of use and could be used in the field. This would enhance the industrial usefulness of the biosensor by giving them the capability of dealing with a wide variety of real-life problems.

1.3 Research Objectives

There are a number of research issues involved in the fulfilment of the aim of this research. The research objectives, which address these issues, are as follows.

- To carry out a literature survey for classification and critical analysis of biosensor techniques for handling three features of biosensor design optimisation problems: multiple objectives, constraints and variable interaction.
- To carry out a literature survey of certain horticultural produce, in order to identify key analytes of interest that could be measured by electrochemical biosensors.

- To develop simplified techniques that can be used in the field for sample extraction.
- To develop a specific biosensor for the detection of L-malic acid. Also to optimise the biosensor for in field use.
- To compare the performance of the L-malic acid biosensor with existing commercially available test kit methods.
- Develop biosensors for other analytes, which include L-ascorbic acid, L-glutamic acid, L-amino acid, D-glucose. To validate the performance of the proposed biosensors with commercially available test kits.
- To test the biosensors on specific horticulture produce.
- To develop a multi analyte biosensor, which is able to measure glucose, ascorbic acid, and malic acid simultaneously on a single electrode pad.

1.4 Thesis Layout

The layout of this thesis is developed based on the story of this research. This story, which is pictorially depicted in Figure 1.5, aids the identification of individual chapters. A brief description of these chapters is given below.

Chapter 1 discusses the background of this research. It presents the problem statement and motivation for this research and describes the concept of biosensor, in particular their relationship to the food industry.

Chapter 2 provides a survey of literature in the area of biosensors. The chapter is divided into individual sections, where each individual analyte of interest is discussed.

Chapter 3 provides a survey of literature in the area of individual fruits and vegetables. It presents a critical analysis of these horticultural produce in terms of the effect of individual analytes on the quality of apples, potatoes, and tomatoes.

Chapter 4 includes non-biosensor methods for determination of parameters of interest. This chapter explores the tools for sample juice extraction that could be used in the field with the developed biosensor. It further gives results of taste panel analysis of six varieties of tomatoes. Key analytes are measured in tomatoes and the results are subjected to Principal Component Analysis (PCA).

Chapter 5 develops biosensor techniques for measuring L-malic acid. This chapter compares two methods for measuring the analyte. It also compares real sample results of the biosensor with a commercially available test kit method.

Chapter 6 includes development of individual biosensors for L-glutamic acid, L-amino acids, L-ascorbic acid and also for D-glucose. The biosensors are tested with real samples and the results are compared with commercially available test kits.

Chapter 7 this chapter discuses the development of a multi-analyte biosensor, which is able to measure D-glucose, L-ascorbic acid, and L-malic acid, simultaneously on a single screen printed sensor.

Chapter 8 concludes this thesis by stating general results and future work.

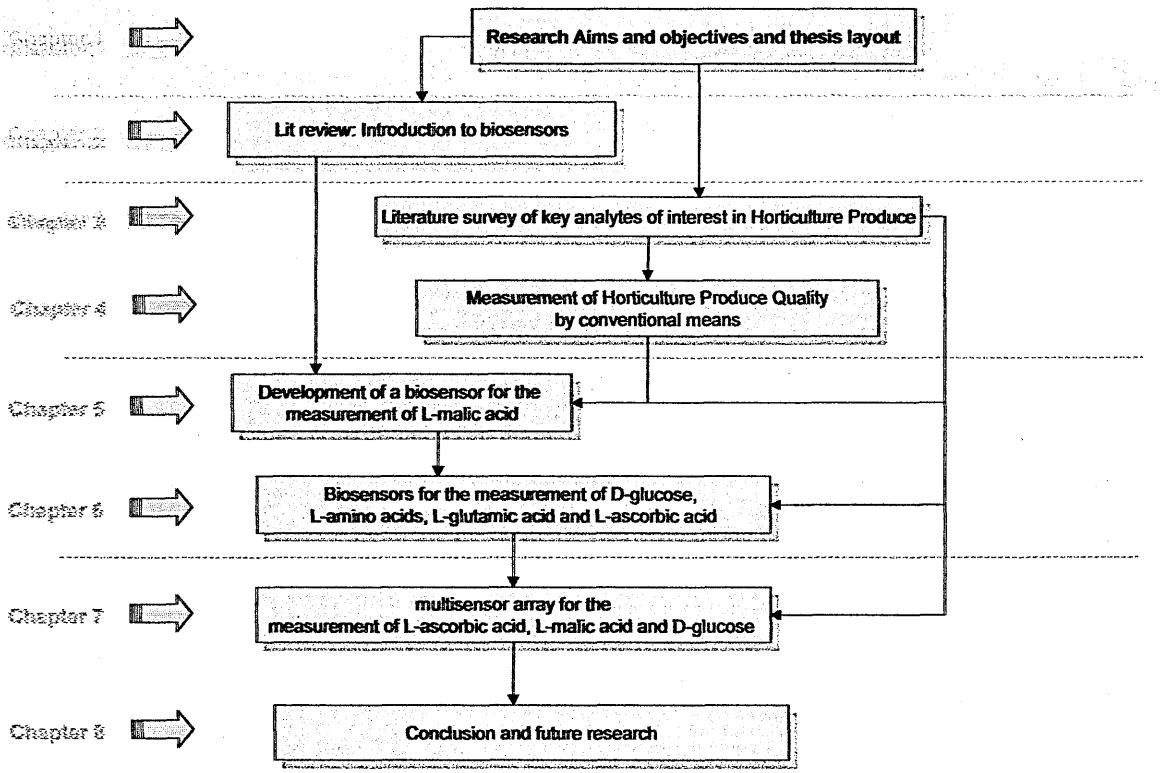


Figure 1.1: Thesis Layout

2 A REVIEW OF LITERATURE

2.1 Introduction to biosensors

For a full understanding of biosensor operations, it is essential to have knowledge of the theory of biosensors. It is because of this reason that some basic concepts of biosensors that are relevant for subsequent analysis are discussed briefly in this chapter.

2.1.1 Principles of biosensors

This Section defines a biosensor and explains the various types. It also describes the concepts involved in amperometric biosensors.

2.1.1.1 Definition of a biosensor

A biosensor may be defined as an analytical device incorporating a biological sensing element, which is either intimately associated with or integrated within a transducer (Turner, 1987).

More recently the final IUPAC definition of electrochemical biosensors has been produced (Thevenot *et al.,* 2001) as follows. An electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitive or semi-quantitive analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with an electrochemical transducer. The biological sensing elements that have been applied to biosensors have been wide ranging and include enzymes, antibodies, receptors, membranes, tissue, cells and organelles. When applied to a variety of physical signal

transduction techniques such as electrochemical, optical, piezoelectric, acoustic and calorimetric methods, the diversity, divisions and sub-divisions of biosensors are vast (Newman and Turner, 1994).

2.1.1.2 Operation of a biosensor

The basic concept of a biosensor is often related to the original Clark and Lyons glucose biosensor (1962), where in essence, immobolised biological component is used to effect a chemical change, which can be converted into an electrical signal by means of an associated transducer. The electrical output can then be displayed as a quantifiable value relating to the amount of analyte present in the test solution (Figure 2-1).

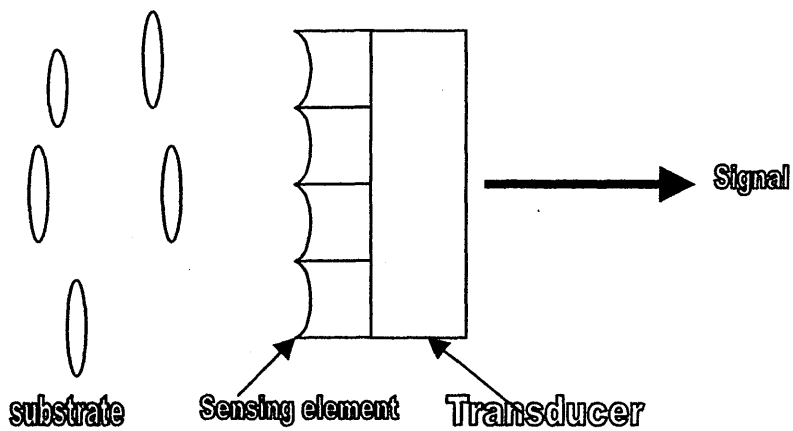


Figure 2.1 Diagrammatic view of a biosensor

However, over the last 30 years, extensive research in biosensors has led to the examination of a wide range of transducers and their subtypes (Figure 1-2). A full explanation of each of the transducers is available elsewhere (Turner, 1987) and only

a brief discussion of the transducers relevant to the measurement of horticulture produce is provided.

2.2 Non-electrochemical biosensors

2.2.1 Optical biosensor

An optical biosensor is based on a biological sensing element linked intimately to an optical transducer. The transducer is able to measure the change in optical properties when the sensing element interacts with the target molecule. There are various types of optical transducers e.g. absorption, fluorescence (Tothill 1997).

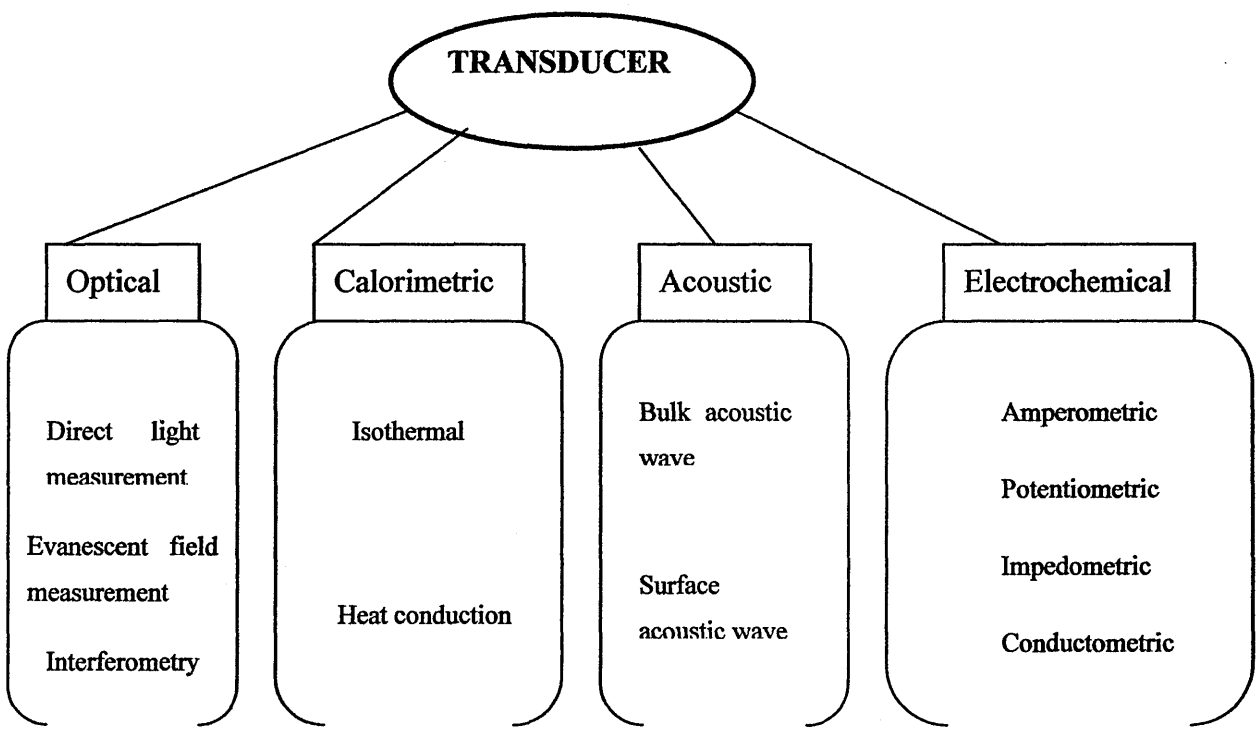


Figure 2.2 Transducers used in biosensors

NAD(P)/NAD(P)H dehydrogenase enzymes are obvious candidates for linking to optical transducers since NAD(P)H is known to absorb light at 340 nm and fluoresce at 460 nm (Luong 1997). However no papers appear in the literature that relate to the specific detection of malic acid using optical transducers. This could be due to the expense of acquiring NAD(P)/NAD(P)H, and also due to the instability of these co-factors. Optical sensors have been used to detect other compounds that are present in horticultural produce e.g. a fluorometric biosensor has been constructed to analyse glucose and fructose (Lee *et al*, 1994).

2.2.2 Calorimetric biosensor

Calorimetric biosensors are Thermometric devises that are able to measure the change in enthalpy when a biological reaction is occurring. These biosensors can function by immobilising the biocomponent of interest into a small column, which is in close proximity to the heat sensing transducer of which a thermister is the most common type. It is possible for thermal biosensors to measure a wide range of annalytes since nearly all enzyme catalysed reactions release a considerable amount of heat energy i.e. 20-100 kJ mol^{-1} (Mosbach 1991). Commercial thermometric biosensors like the ThermoMetric Inc biosensor (Jarfalla, Sweden) have been made, which claim that they have a substrate detection limit of 10^{-5} M. This is useful in the horticultural industry since several food components are present in this range, however the use of sophisticated and expensive instrumentation is a major draw back to these types of biosensors.

2.2.3 Acoustic biosensor

Piezoelectric transducers are acoustic devises that rely on the change in the resonant frequency of wave propagation through a piezoelectric material. These principles can be used to measure mass, viscosity or density changes at the sensor surface. These devises are able to generate and transmit acoustic waves in a frequency dependent manner. Bulk wave devices operate by transmitting a wave from one side of the crystal to the other, while surface acoustic wave (SAW) transmit waves along a single crystal surface (Tothill and Turner, 2003). The use of piezoelectric biosensors in the horticultural industry has been hampered due to the poor sensitivity and also non-specific binding of these sensors (Wagner and Guilbault, 1993).

2.2.4 Magnetic biosensors

Very recently, there have been a number of publications involving biosensors with magnetic transducers. An example of this includes Hall effect transducers, which have been used to detect magnetic labels in immunosensors (Tothill and Turner, 2003). Practical application of these biosensors has been limited, but they have been included for completeness.

2.3 Electrochemical Biosensors

There are various types of electrochemical biosensors that include amperometric sensors that are able to measure the current with a fixed applied voltage, or potentiometric biosensors that are able to measure the voltage when no potential is applied. Conductimetric biosensors are able to measure the net change in conductance during many enzyme catalysed reactions, while impedance devises are

able to measure the total electrical resistance to the flow of an alternating current being passed through a given medium. Impedance devices and conductometric devices lack of specificity for a given analyte makes them difficult to be useful for the horticulture industry.

Amperometric biosensors generally detect analytes by measuring the presence of electroactive species such as oxygen, hydrogen peroxide and NADPH. Potentiometric biosensors generally detect analytes by measuring compounds such as carbon dioxide or hydrogen ions (pH). Amperometric biosensors combined with oxidoreductase enzymes have received much attention since the enzymatic reaction may be measured both easily and sensitively (Luong 1988). The most successful commercial biosensor to date (MediSense, Inc U.S.), has been an amperometrically based glucose biosensor (which makes use of mediators), for use by diabetic patients in the home (Higgins *et al* 1985., Magner 1998). Mediators are commonly used with redux enzymes, because of their ability to regenerate co-substrates (Schmidt *et al.*, 1997). An example can be seen from the reaction scheme below where the reduced FAD (flavin adenine dinucleotide), which is the prosthetic group of glucose oxidase, is oxidised by the mediator (M), which is thus reduced (M`). Oxidation at the electrode surface regenerates the mediator.

$$\text{D-glucose} + \text{FAD} \xrightarrow{\text{Glucose oxidase}} \text{gluconolactone} + \text{FADH}_2$$

$$\text{FADH}_2 + 2\text{M} \longrightarrow \text{FAD} + 2\text{H}^+ + 2\text{M}`$$

$$2\text{M}` \longrightarrow 2\text{M} + 2\text{e} \text{ (electrode surface)}$$

Amperometric biosensors are ideal for commercialisation because they may have the following features:

- Low cost manufacture of electrodes
- High degree of reproducibility even with one shot sensors
- Hand held potentiostat devices

The Medisense range of biosensors have been commercially viable because they have made use of thick-film technology (Nagata *et al* 1995). This technology which includes screen-printing, can help offer the possibility for low cost single use biosensors. Advantages include the avoidance of cross-contamination and minimisation of problems concerned with electrode fouling. One of the most useful characteristics of screen-printed electrodes is their versatility. Many different types of inks can be deposited onto numerous substrates and base inks may be mixed with modifiers as required. These electrodes can also be mass-produced where each electrode may only cost a few pence (Magner 1998), and the data gained from these electrodes is also reproducibly acceptable for the intended usage. In the case of amperometric sensors a three-electrode configuration is normally used which consists of the working counter and reference electrodes (Figure 2-3).

The reference electrode is fabricated using Ag/AgCl while the working and counter electrodes are typically carbon based. One of the major disadvantages of using bare carbon for the working electrode is that the potential needed to detect some products can be high. The working electrode therefore contains a commercially available rhodinized carbon (MCA4, Cambridge, UK). The rhodinized carbon has advantages

over other working electrode materials e.g Platonised carbon which is highly electrocatalytic but not as specific as rhodenised carbon to certain substrates (Newman et al 1995).

2.4 Theory of amperometric sensors

Amperometric techniques have been most useful in quantitative analysis because they offer high sensitivity and make use of equipment that is simple to operate. Amperometric biosensors are usually dependent on enzymes. This is because enzymes are able to catalyse electrochemically non-active analytes into products, which can be oxidised or reduced at the working electrode.

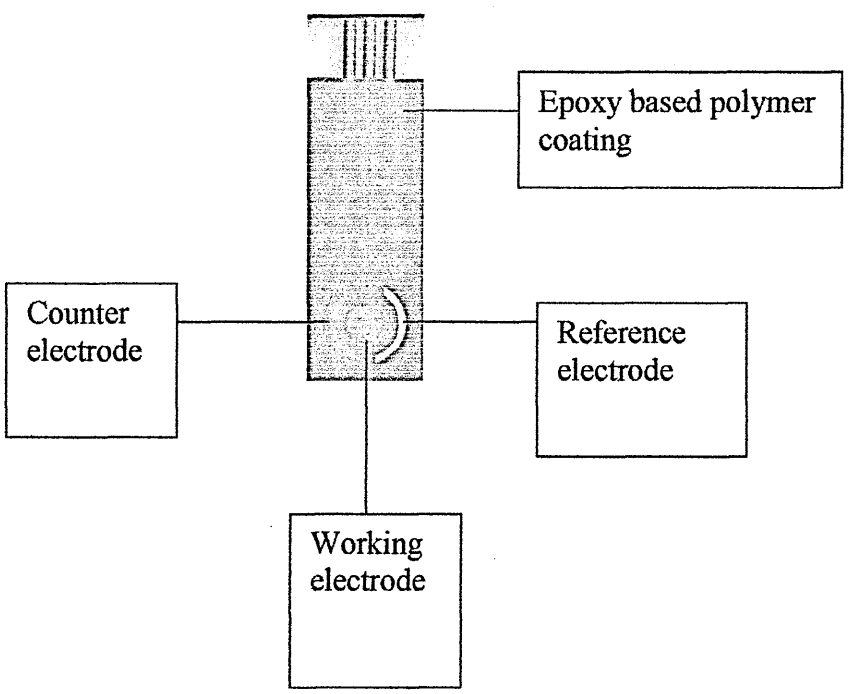


Figure 2.3 Schematic of a typical screen printed electrode

By measuring the current, it is possible to determine the concentration of the analyte of interest, since the current produced is proportional to the electroactive product and the concentration of this product is proportional to the concentration of the analyte of interest. This section introduces theoretical principles to amperometric biosensors

2.4.1 Amperometry

The thermodynamic requirement for a cell reaction to take place at the electrode surface is that the overall Gibbs Free Energy change must be negative. The principle of amperometery, in detecting species in solutions is based on the use of electrolytic cells where a voltage is required to bring about a physical or chemical change on the electrode surface (Somasundrum, 1994).

From the Nernst equation (shown below) it can be seen that the potential difference is measured by subtracting the potential of the solution interface from the potential of the metal electrode. Practically this is not possible because the voltmeter can only measure the potential difference by passing a current in the nano-ampere range, and therefore a circuit needs to be formed (Fisher, 1996). A counter electrode is used to form this circuit and help pass the current from the metal electrode to the sample and via the counter electrode back to the voltmeter (Figure 2.4).

$$E=E_0 +(RT/nf)\ \ln a_1 \quad \text{.....(1)}$$

Where:

E_0 = standared potential for a_1

a_1 = ionic species of interest in mol l^{-1}

R= gas constant

f = Faraday constant

T = Temperature in Kelvins

n = total number of charges on an ion.

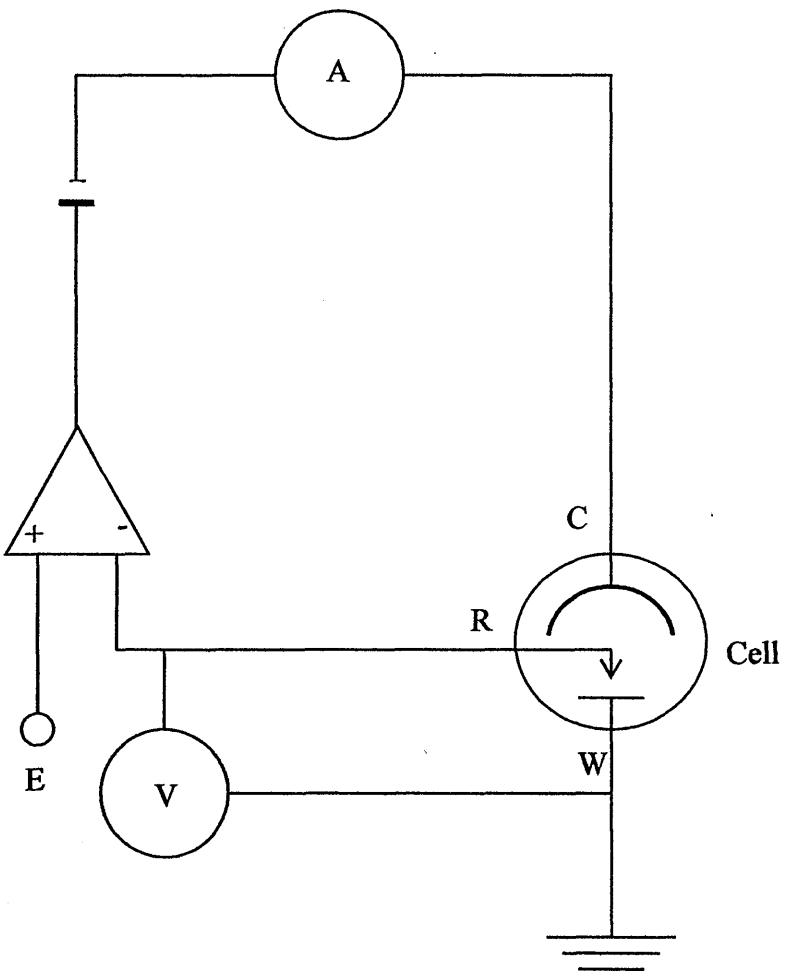


Figure 2.4 Circuit diagram for a 3 electrode electrochemical biosensor

Amperometric biosensors usually contain a third reference electrode (Figure 2.4). This is to make sure that the potential difference applied between the reference electrode and working electrode is constant, while the current is driven between the working and counter electrodes. The current is prevented from passing through the reference electrode in order to help reduce the IR drop (where the solution has a resistance R resulting from the current I flowing through the solution). The reaction at the electrode is only able to take place if the reference electrode has:

- A fixed chemical composition as well as the solution in which the electrode is exposed e.g. for the Ag/AgCl reference electrode 0.1 M of KCl (potassium chloride) is dissolved in the solution phase.
    - *This will keep the potential of the reference electrode constant since the change in concentration of chlorine ions in solution could result in the overall potential difference to change.*
- A large electrical current should not pass through the reference electrode since electrolysis may take place and the reference electrode would not be able to maintain its potential

2.5 Individual biosensors

As mentioned in chapter one section (1.4) the work in this thesis entails the need to measure individual analytes in order to ascertain the overall quality of horticulture produce. Therefore this section specifically addresses current research for key analytes that have been used with biosensors previously.

2.5.1 Glutamate biosensor

Alvarez-Crespo *et al.*, (1997) developed a glutamate biosensor by joining the electrocatalytic NAD(P)H oxidation at electropolymerizing *o*-phenylenediamine film (PPD) together with the enzymatic oxidation of glutamate catalysed by a glutamate dehydrogenase enzyme (GIDH). The preparation of PPD films was carried out by

Table 2.1 Linear ranges of glutamate biosensors

Type of biosensor approach	Linear range	Authors
Electrochemical	5 μM to 78 μM	Alvarez-Crespo *et al.*, (1997)
Electrochemical	0.02 mM to 2.0 mM	Ye Bang-Ce *et al.*, (1995)
Optical	0.02 mM to 1.0 mM	Dremel and Schmid, (1991)
Optical	0.1 mM to 2.5 mM	Dremel and Schmid, (1991)
Electrochemical	0.1 μM to 100 μM	Karyakin *et al.*, (2000)
Electrochemical	0.3 μM to 800 μM	Pan *et al.*, (1996)
Electrochemical	50 μM to 1.3 mM	Amine and Kauffmann, (1992)
Electrochemical	0.5 μM to 50 mM	Ling *et al.*, (2000)
Optical	1 mM to 60 mM	Pantano and Kuhr, (1993)
Electrochemical	0.1 mM to 1.5 mM	White *et al.*, (1994)
Electrochemical	0.1 μM to 10 μM	Liu *et* al., (1999)
Electrochemical	2.6 μM to 0.8 mM	Almeida and Mulchandani, (1993)

potential scan electropolymerization in an aqueous solution using (GIDH)-NAD(P)-modified carbon paste electrodes. This conducting film allows the oxidation of the

enzymatically produced NAD(P)H at applied potentials as low as 0 V (AgCl). They were able to achieve a linear range between $5{\cdot}0 \times 10^{-6}$-$7{\cdot}8 \times 10^{-5}$ M with a detection limit of $3{\cdot}8 \times 10^{-6}$ M.

Ye Bang-Ce *et al.*, (1995) were able to isolate glutamate oxidase from *Streptomyces* P-106. They were able to form a micro-enzyme electrode by cross linking the purified glutamate oxidase, with glutaraldehyde on an aminopropyl-platinized-platinum wire (ø 0.5 mm). By use of a flow injection analysis system Ye Bang-Ce *et al.*, (1995) were able o show a linear range between 0.02 mol l^{-1} to 2.0 mmol l^{-1}. The system showed linear response to the L-glutamate concentration, ranging from 3.0 mg l^{-1} to 300 mg l^{-1} (approx. 0.02 mol l^{-1} to 2.0 mmol l^{-1}).

Dremel and Schmid, (1991), were able to make use of a flow-injection system for the fibre-optical determination of L-glutamate in food and pharmaceutical preparations. They were able to compare two types of fibre-optic biosensors. The first type was an oxygen-sensitive optrode and was covered with a membrane onto which was immobilized L-glutamate oxidase. This biosensor was able to measure the decrease in oxygen partial pressure in the presence of glutamate as a result of enzymatic reaction, which was determined via dynamic quenching of the fluorescence of an oxygen-sensitive indicator dye. This type of glutamate biosensor showed a linear response from 0.02 to 1.0 mM. As a comparison, a carbon dioxide-sensitive optrode was covered with a membrane of immobilized L-glutamate decarboxylase. The production of carbon dioxide in the presence of substrate was determined via the changes in the pH of a carbon dioxide sensor consisting of a membrane-covered pH-sensitive fluorescent pH indicator dye entrapped in a hydrogencarbonate buffer. The

carbon dioxide optrode-based glutamate biosensor was able to show a linear response from 0.1 to 2.5 mM glutamate.

Karyakin *et al.*, (2000), were able to immobilise glutamate oxidase on to the surface of the Prussian Blue-modified electrode in a Nafion layer. The Prussian blue was able to act as a transducer in selectively detecting the formation of hydrogen peroxide reduction in the presence of oxygen. This methodology was able to reduce the influence of reductants by using the low potential of (0.0 V Ag/AgCl). The linear range was between 1×10^{-7} to 1×10^{-4} M, while the limit of detection was 1×10^{-7} M.

Pan *et al.*, (1996), were able to report on a glutamate biosensor constructed with a film of Nafion between the platinum anode and a layer of immobilized glutamate oxidase. Ideal sensor response properties were shown to be obtained with 0.91 units of immobilised enzyme operating at pH 8 and at 48 °C. They were able to report linearity up to 800 μM and a limit of detection of 0.3 μM. The Nafion membrane was reported to enhance the selectivity for glutamate over electroactive species such as ascorbic acid, but could not totally remove interferent signals when glutamate levels were ten-times less than ascorbic acid.

Amine and Kauffmann, (1992) incorporated the glutamate oxidase into the electrode matrix of the carbon paste. The biosensor was characterized using hexacyanoferrate(III) as the electrochemical mediator and phenazine methosulphate as the enzyme (coenzyme) mediator. Octadecylamine was mixed into the enzyme modified carbon paste, in order to enhance the electrochemical properties of hexacyanoferrate(II)/(III). Linear range was between 50 μM and 1.3 mM glutamate.

Zhang and Tan, (2001) were able to make use of AFM in studying the surface topography of immobilized glutamate dehydrogenase on two-dimensional glutamate biosensor surfaces. Surface analysis had revealed that the enzymatic activity of the immobilized GDH molecules on the biosensor surface is linked to surface roughness. Fractal dimension of the immobilization sensor surface was found to be a good parameter for judging the quality of the immobilized biosensors. They were able to conclude that as enzyme immobilization time increases, the biosensor has its maximum activity with around 18 h of immobilization in 10^{-6} M GDH solution.

Pantano and Kuhr, (1993) have fabricated a microbiosensor by immobolising glutamate dehydrogenase on a carbon fiber microelectrode surface via avidin-biotine technology, and achieved a detection limit of 0.5 mM and a 1 to 60 mM linear concentration range.

Ling *et al.*, (2000) described a biosensor for the detection of glutamate by use of the enzyme L-glutamic decarboxylase. The biosensor consisted of an enzyme column reactor of L-glutamic decarboxylase immobilized on a novel ion exchange resin (carboxymethyl-copolymer of allyl dextran and *N.N'*-methylene-bisacrylamide CM-CADB) and ion analyzer coupled with a CO_2 electrode. The optimum pH of the enzyme was found to be 5.5 while the optimum temperature was 48°C. The limit of detection of the biosensor was found to be 1.0×10^{-5} M. The linearity response is in the range of 5×10^{-2} - 5×10^{-5} M.

White *et al.*, (1994) were able to develop an amperometric biosensor for glutamate using screen-printed electrodes. Biosensors incorporating glutamate oxidase were

fabricated based on glutaraldehyde immobilization. A linear range extended from 0.1 mM to 1.5 mM.

Most GluDH-based sensors are based on the detection of the reduced form of nicotinamide adenine dinucleotide (NADH), by electrochemical oxidation. However, the electrochemical oxidation process is slow because the reaction involves the concerted transfer of two electrons and a proton. Moreover, a high overpotential is required to achieve practical rates for most of the electrodes. The other problems are electrode fouling caused by the oxidation of NADH and the inhibition of the major product, NAD^+, formed in the direct electrochemical oxidation process (Blaedel and Jenkins, 1975; and Blankespoor and Miller, 1984).

To solve these problems, one approach designed to accelerate the oxidation is the use of an enzymatic reaction coupled with an electron transfer mediator such as a ferrocene ferrocyanide complex. Another approach is to oxidise NADH using NADH oxidase or diaphorase. NADH oxidase is a highly stable and pH-insensitive flavoenzyme compared with diaphorase (McNeil *et al.*, 1989), and it also accepts oxygen as an electron acceptor (Park *et al.*, 1992). Liu *et al* (1999) have developed a biosensor consisting of a glassy carbon electrode modified with an osmium-polyvinylpyridine-based bottom layer containing horseradish peroxidase, and a bovine serum albumin (BSA)-gluteraldehyde top layer containing glutamate dehydrogenase and NADH oxidase. The detectable glutamate concentration range was from 0.1 to 10 μM and the detection limit was 0.1 μM. The potential used was 0 V against Ag/AgCl.

Almeida and Mulchandani, (1993) developed a sensor that is based on a carbon paste electrode containing the electron transfer mediator tetrathiafulvalene (TTF), and the enzyme L-glutamate oxidase immobilized on the electrode surface. The enzyme is crosslinked with glutaradehyde and the matrix held on the electrode surface using an electrochemically deposited polymer. Tetrathiafulvalene is shown to efficiently reoxidize the reduced flavin adenine dinucleotide centres of glutamate oxidase, permitting operation of the sensor at low applied potentials. The electrode, when operated at +0.15 V (vs. Ag/AgCl), responded linearly to L-glutamate concentration up to 0.8 mM, the lower detection limit being 2.6 μM, with a response time of 2 min. The sensor was used for the determination of L-glutamate in food samples, with excellent correlations achieved when compared to results obtained by enzymatic analysis using glutamate dehydrogenase.

Wang and Arnold, (1992) developed dual-enzyme fiber-optic biosensor for glutamate. An enzyme layer composed of glutamate dehydrogenase (GDH) and glutamate-pyruvate transaminase (GPT) is used to produce reduced nicotinamide adenine dinucleotide (NADH) at the tip of a fiber-optic probe. NADH luminescence is monitored through this probe and the measured fluorescence intensity is related to the concentration of glutamate. GDH catalyzes the formation of NADH, and GPT drives the GDH reaction by removing a reaction product and regenerating glutamate. The optimum response was obtained at pH 7.4 while the detection limit was 0.13-μM. Glutamine and lysine responded slightly to the biosensor.

2.5.2 Glucose Oxidase:

Initially Clark and Lyons developed the first glucose biosensor in 1962. Clark and Lyons entrapped glucose oxidase physically against the electrode with a piece of dialysis membrane. They reasoned that they could follow the activity of the enzyme by following the changes in the oxygen concentration. Thus the first known biosensor was developed. Today glucose oxidase biosensors are used by millions of Diabetics around the world.

Glucose Oxidase continues to be the most common enzyme used in biosensor analysis.

1) It is easy and cheap to obtain, (the enzyme is a secretion produced by certain soil-living fungi).

2) It is one of the most robust enzymes around (it withstands greater extremes of pH, ionic strength, temperature than many other enzymes), thus allowing less stringent conditions during the manufacturing process and also relatively carefree storage and use by the home-user of the biosensor.

3) The concentration range of glucose with which GOD reacts optimally happens to coincide with the range of concentrations encountered in human blood.

Many glucose oxidase biosensors detect Hydrogen peroxide or Oxygen as the product of the glucose reaction. The following reaction scheme applies:

β-D-Glucose + Enzyme-FAD ------> Enzyme-$FADH_2$ + D-Gluconolactone

Enzyme-$FADH_2$ + O_2 ------> Enzyme-FAD + H_2O_2

Due to the excessive number of publications on the glucose biosensor, only the most recent publications relating to food will be covered in this section. A summery of the linear ranges achieved by various authors is shown in Table 2.2.

Table 2.2 Linear ranges of D-glucose biosensors

Type of biosensor approach	Linear range	Authors
Calorimetric	0.2 mM to 1 mM and 10 mM to 50 mM	Ramanathan *et al.*, (2001)
Electrochemical	9.9 mM to 100 mM	Palmisano *et al.*, (2000)
Electrochemical	1 mM to 10 mM	Volotovsky and Kim, (1998)
Electrochemical	100 mM with microporous polycarbonate outer membrane 2000 mM with mixed PVC/polycarbonate membrane	Maines *et al.*, (1996)
Optical	0.1 mM to 500 mM	Dremel *et al.*, (1989)

Ramanathan *et al.*, (2001) report a sol–gel (SG) based glucose biosensor using thermometric measurement. The enzymes (glucose oxidase, GOD and catalase, CAT) were entrapped on the surface of reticulated vitreous carbon cylinder (RVC cartridge) using SG as a binder. This 'RVC cartridge' was placed within the column of an enzyme thermistor (ET) device. Injection of various D-glucose concentrations resulted in changing the heat content of the circulating buffer, recorded as a

thermometric peak by a sensitive thermistor. Independent calibration curves between 10 and 50 mM and between 0.2 and 1 mM D-glucose was obtained by plotting the D-glucose concentration versus the thermometric peak height. Electroactive interferents, aspartic acid, uric acid, glutamic acid and urea in 0.1mM concentrations were injected. There was no effect in the signal response. The results were compared with commercial juice samples.

Palmisano *et al.*, (2000) developed a biosensor to measure glucose in tomato juice. The biosensor is based on a conventional thin layer flow-through cell equipped with a Pt dual electrode (parallel configuration). Each Pt disk was modified by a composite bilayer consisting of an electrosynthesised overoxidized polypyrrole (PPYox) anti-interference membrane covered by an enzyme entrapping gel, obtained by glutaraldehyde co-crosslinking of glucose oxidase. The PPY_{OX} is reported to have excellent interference-rejection capabilities. The linear range for the biosensor was between 9.9 mM to 100 mM. The potential used in the experiment was 0.7 V (Ag/AgCl).

Volotovsky and Kim, (1998) developed an ion sensitive field-effect transistor (ISFET) based biosensor for sequential determination of glucose. A sensor chip, containing ISFET was covered by GOD-urease mixture. An in house manufactured, ISFET controller had been used for measurement of pH variations in the biomembranes. The pH changes induced by a spontaneous hydrolysis of D-glucono-d-lactone to gluconic acid were registered with an ISFET. The linear range for the probe was between 1 and 10 mM.

Maines *et al.*, (1996) employed a series of microporous and homogeneous outer membranes in an amperometric glucose oxidase enzyme electrode for the determination of glucose at concentrations substantially higher than the enzyme k_M. They were able to show that by reducing the porosity of microporous polycarbonate by use of low pore diameter (0.01 μm), and treating the membrane with liquid phase lipid (tripalmitin), the linearity was increased to 100 mM. The linear range was further increased to 500 mM by decreasing the pore size further and by use of silane treatment (Mullen *et al* 1993). Maines *et al*, were able to achieve the highest linearity of 2000 mM with a mixed PVC/polycarbonate membrane.

Dremel *et al.*, (1989) Developed a flow-injection glucose biosensor based on an oxygen optrode with immobilized glucose oxidase (GOD). The consumption of oxygen was determined via dynamic quenching of the fluorescence of an indicator by molecular oxygen. The system is linear for 0.1–500 mM glucose, with a Coefficient of Variation (CV) value of 2%. This biosensor was useful in analysing 60 samples per hour and the sensor's shelf life was 400 hours.

2.5.3 Ascorbic acid:

L-ascorbic acid is one of the most important vitamins for human health (Tolbert and Ward 1982). Measurement of L-ascorbic acid using biosensors is predominatly performed with the enzyme ascorbic oxidase (Volotovsky and Kim, (1998)). Nakamura and colleagues (1968), were able to propose the following mechanism for ascorbic oxidase catalysing the oxidation of L-ascorbic acid in the presence of molecular oxygen:

$$X_{ox} + Asc \underset{K-1}{\overset{K+1}{\rightleftarrows}} X_{ox} . Asc \xrightarrow{K+2} X_{red} + R$$

$$X_{red} + O_2 \underset{K-3}{\overset{K+3}{\rightleftarrows}} X_{red} . O_2 \xrightarrow{K+4} X_{ox} + H_2O$$

where, X_{ox} and X_{red} represent the oxidized and reduced formsascorbate oxidase, respectively; Asc stands for the ascorbate ion (primary substrate) and P is ascorbate free radical. This section deals with biosensor stratagies for measuring L-ascorbic acid. Table 2.3 is able to depict the linear ranges of various biosensors that are discussed.

Table 2.3 Linear range of L-ascorbic acid biosensors

Type of biosensor approach	Linear range	Authors
Electrochemical	62.5 µM to 500 µM	Marques and Lima Filho, (1992)
Electrochemical	0.25 mM to 1.6 mM	Uchiyama and Umetsu, (1991)
Electrochemical	0.02 mM to 0.57 mM	Macholán and Chmelíková, (1986)
Electrochemical	8 µM to 0.45 mM	Fernandes *et al.*, (1999)
Electrochemical	0.5 mM to 2 mM	Volotovsky and Kim, (1998)

Marques and Lima Filho, (1992) have developed a biosensor for the detection of L-ascorbic acid using the enzyme ascorbate oxidase. The enzyme was extracted from Cucurbita maxima, and immobilized by covalent bonding, using glutaradehyde as a bifunctional agent, on alkylamine glass beads; oxygen electrode was applied as a transducer. The system has sensitivity from 62.5μM up to 500 μM of ascorbic acid with satisfactory operation for more than 2 months. Uchiyama and Umetsu, (1991) measured L-ascorbic acid by concentration-step amperometry using a thin layer of carbon felt impregnated with cucumber juice as an enzyme solution of ascorbate oxidase. The dilute fruit juice was added on top of the carbon felt and the decreased current peak caused by the enzymatic reaction was measured. The cucumber juice was prepared by filtration and centrifugal separation. The peak current was proportional to the concentration of L-ascorbic acid in the concentration range 2.5×10^{-4} to 1.6×10^{-3} M.

Macholán and Chmelíková, (1986) also coupled a slice of cucumber *Cucumis sativus*) to a Clark-type oxygen electrode. The amperometric method was based on monitoring the decrease in the current of oxygen at an applied potential of -650 mV vs. Ag/AgCl. They were able to obtain a linear range of 0.02 mM to 0.57 mM. The biosensor is highly selective towards ascorbic acid with a response time of 70-90 s.

Volotovsky and Kim (1998) utilised the properties of Peroxidase which are able to reduce hydrogen peroxide to water in the presence of some hydrogen donors, and this enzymatic reaction can be used for the detection of either hydrogen peroxide or donor species. One of these doner species is L-ascorbic acid, which is converted to dehydro-L-ascorbic acid during the peroxidase-catalyzed reaction. During the

conversion process the enzyme peroxidase is immobolised in a 'biomembrane' on an ion-sensitive field effect transistor. Any pH changes on the 'biomembrane' could be detected with the ion-sensitive field effect transistor. Conversion of L-ascorbic acid into dehydro-L-ascorbic acid considerably increases the pH of the 'biomembrane'. Biosensor results for ascorbic acid were compared with High Performance Liquid Chromatography results for fruit juice; a high degree of correlation was achieved.

The reaction scheme is shown below:

$$\text{Ascorbic acid} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{Dehydroascorbic acid} + 2H_2O$$

Dynamic range of the probe was 0.5 mM to 2 mM.

Fernandes *et al.*, (1999) developed a potentiometric biosensor for L-ascorbic acid based on ascorbate oxidase. The principle for the biosensor was based on the ability of L-ascorbate oxidase to be bound in three different chemical complexes forming the active sites. Where the potential variation is caused by the reduction on the enzyme of Cu^{2+} to Cu^{1+}, which is due to the presence of ascorbate ion, changing the electronic density on the electrode surface, which is measured by the potentiomeric transducer. The enzyme was extracted of the epicarp of *Cucumis sativus* L. fruit. Few potentiometric electrodes sensitive to L-ascorbic acid were developed due to the difficulties in the preparation of sensitive membranes and the low selectivity. Fernandes *et al.*, tried to overcome the problem by immobolising the enzyme in a

graphite/epoxy electrode by occlusion in a poly (ethylene-co-vinyl acetate) matrix. The linear range for the biosensor was 8.0×10^{-6} mol l^{-1} to 4.5×10^{-4} mol l^{-1}.

2.5.4 Malic acid biosensor

Two enzymatic pathways have been used for malic acid analysis. These pathways include NAD^+ dependent malate dehydrogenase or $NADP^+$ dependent malate dehydrogenase (malic enzyme). The following Section reviews both pathways. The linear ranges of various malate biosensors that discussed in the context are shown in table 2.4.

Table 2.4 Depicts the linear ranges of various malic acid biosensors.

Type of biosensor approach	Linear range	Authors
Electrochemical	0.25 mM to 2.0 mM	Silber *et al.*, (1994)
Electrochemical	0.02 mM to 1.0 mM	Matsumoto *et al.*, (1995)
Electrochemical	2 µmol and 0.9 mM and 2 µmol and 1 mM	Gajovic *et al.*, (1997)
Electrochemical	0.01 mM to 1.2 mM	Gajovic *et al.*, (1998)
Electrochemical	0.02 mM and 1 mM	Matsumoto *et al.*, (1996)

Silber *et al.*, (1994) has been able to measure malic acid by using the enzyme malate dehydrogenase. This was achieved by immobilising the enzyme by an entrapment method, in a polymer matrix. The pH optimum of the enzyme was found to be 7, but

the immobilised malate dehydrogenase became unstable after a few days. The linear range for the enzyme was between 0.25-2.0 mM.

Matsumoto *et al.*, (1995) measured malic acid concentration in fruit by having a flow injection system. Malate dehydrogenase and NADH oxidase were immobilised on APCPG (Aminopropyl controlled-pore glass). The following reaction scheme was used:

$$\text{L-malate} + NAD^+ \xleftrightarrow{\text{Malate dehydrogenase}} \text{oxalacetate} + NADH + H^+$$

$$NADH + H^+ + O_2 \xrightarrow{\text{NADH oxidase}} NAD^+ + H_2O_2$$

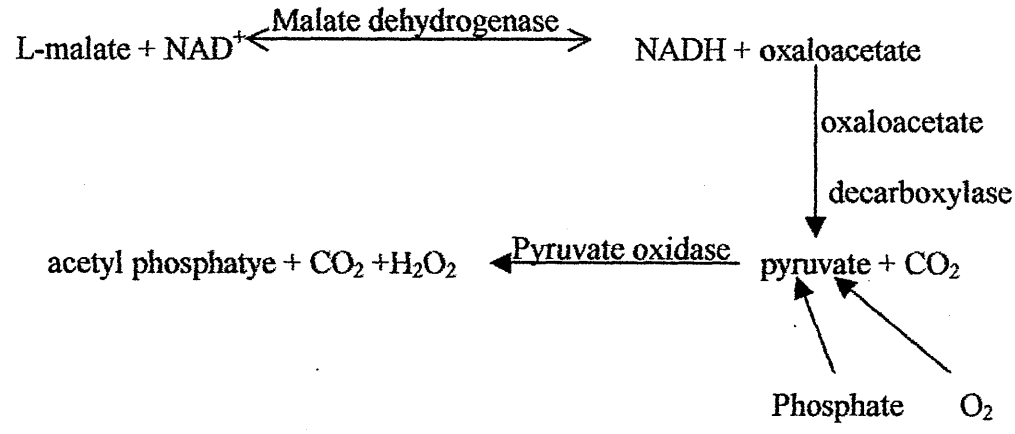
The hydrogen peroxide was measured using an amperometric electrode system at a potential of 0.6V vs.Ag/AgCl reference electrode. It was found that the optimum pH for the enzyme was between 9.2 and 9.5. The linear range for the enzyme was found to be between 0.02 mM to 1.0 mM.

Gajovic *et al.*, (1997) used both malate dehydrogenase and malic enzyme in two separate reaction schemes (Figure 2.5). In both cases the consumption of oxygen was measured during acetyl phosphate production. Malate dehydrogenase was immobilised with oxaloacetate decarboxylase and pyruvate in gelatine. The same procedure was done with malic enzyme however; oxaloacetate decarboxylase was not used (Figure 2.5).

The results indicated that the malate dehydrogenase curve was seen to be partially non-linear, while the linear range detected was between 2 μmol and 0.9 mM. In the

case of malic enzyme the linear range was recorded between 2 µmol and 1 mM. It was also found that the malic enzyme was more stable then malate dehydrogenase at room temperature (when immobilised in gelatine).

a:



b:

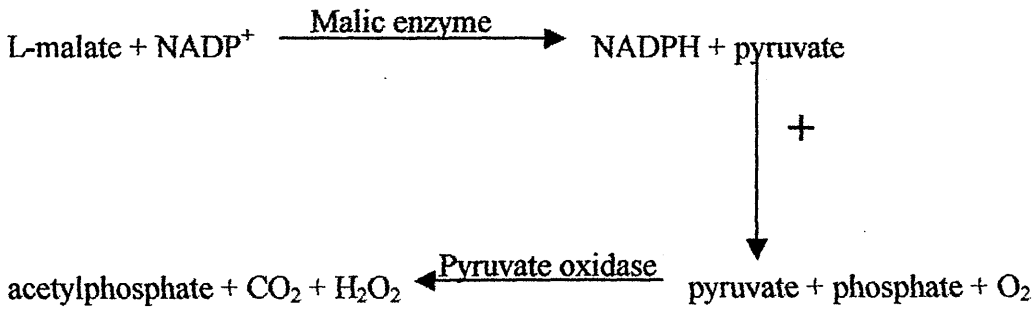


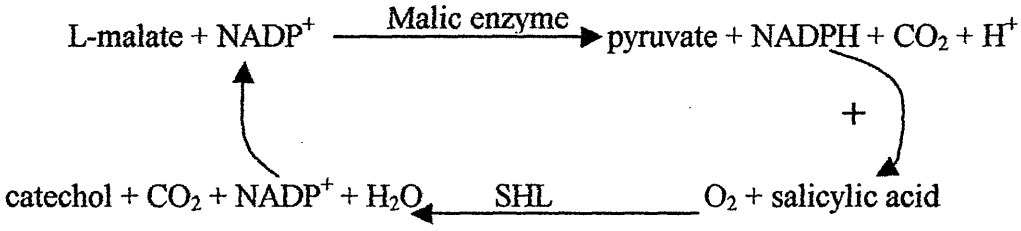
Figure 2.5 Reaction schemes proposed by Gajovic et al, where a: scheme with malate dehydrogenase and b: scheme with malic enzyme.

Palleschietal e*t al.*, (1994) were able to measure malic acid by using the malic enzyme scheme.

$$\text{L-malate} + NADP^+ \xrightarrow{\text{Malic enzyme}} \text{pyruvate} + NADPH + CO_2 + H^+$$

By using a differential pH meter, it was found that the pH variation due to the action of the enzyme on malic acid was proportional to the malic acid present in sample. The linear range for malic enzyme was found to be about 0 to 4 g/l and the buffer pH was 7.4.

Gajovic *et al.*, (1998) was able to make use of the malic enzyme route by coupling the enzyme to salicylate hydroxylase (SHL, E.C. 1.12.13.1). The following reaction scheme was used:



The resultant oxygen consumption was measured by using a Clarke oxygen electrode. Due to the mediated recycling of the $NADP^+$, it was shown that less then 0.025mmol l^{-1} of $NADP^+$ was needed. Also the linear was extended from 0.01 to 1.2 mMol l^{-1} for malic acid. It was also seen that the response did not decrease after 30 days.

Matsumoto *et al.*, (1996) gave a general review of the reaction schemes for measuring malic acid concentration. The malic enzyme route was seen to be advantageous over the malate dehydrogenase route because the optimum pH of the

enzyme was 7.8 compared to 9.5 for malate dehydrogenase. However, it was shown that the malate dehydrogenase and the NADH oxidase route were able to give the highest linear range between 0.02 mmol and 1 mmol. For measuring fruit samples it was seen that reducing sugars in fruit juice did not interfere with the malic enzyme and the malate dehydrogenase /NADH route. Katakis and Dominguez (1997) review the oxidation of NADH (cofactor for malate dehydrogenase). The review also explains various ways of oxidising NADH. This includes diaphorase, which is able to convert NADH to NAD^+.

One of the major reasons why oxidase enzymes are preferred as compared to the dehydrogenase enzymes is the need for these enzymes not to require cofactors such as NADP (Labo *et al.*, 1996). However dehydrogenase enzymes have some advantages over the oxidase enzymes:

- *Oxygen is not involved in the enzymatic reaction and therefore it does not interfere in the reaction step.*
- *The dehydrogenase group is the largest group in the enzyme family and if it was possible to use low potentials for the detection of the analyte, then many more biosensors could made.*

2.5.4.1 Malic enzyme (E.C 1.1.1.40)

As mentioned above malic enzyme uses a cofactor $NADP^+$ to convert L-malic acid to pyruvate.

$$\text{L-malic acid} + NADP^{+} \xrightarrow{\text{malic enzyme}} \text{pyruvate} + CO_2 + NADPH$$

The equilibrium of this reaction favours the formation of pyruvate (Palleschi 1994). Manganese ions or magnesium ions also act as co-factors for this enzyme (Hsuetal 1976). A review by Edwards and Andreo (1992) is able to show that the malic enzyme in plants is able to function as a source of CO_2 for photosynthesis in chloroplasts. The enzyme is also able to show a high specificity and a low km value for $NADP^{+}$.

Malic enzyme can act as a dimer or tetramer depending on its relative pH of 8 or 7 respectively. $NADP^{+}$ and L-malate induce dissociation of the enzyme at pH 8 while magnesium ions are able to induce aggregation of the protein. It was also suggested that the Amino acid residues of the protein had an apparent pka value of 7.7 and therefore needed to be deprotenated in order to stabilise the aggregation of the enzyme to the tetrameric form (Iglesias *et al.*, 1991).

Malic enzyme has also been shown to be naturally present in fruit. Goodenough *et al.*, (1984) suggests that the malic enzyme rise in specific activity at the same time as the specific activities of other enzymes of the Krebs cycle and NAD^{+} mitochondrial malic enzyme (E.C. 1.1.1.39) fall.

It is believed that the increased malic enzyme activity runs in parallel to the decreased activity of malate dehydrogenase (E.C. 1.1.1.37). When the tomato is in the immature green stage, most of the CO_2 is produced by the Krebs Cycle enzymes. However when the fruit reaches the mature green stage only 40% of the CO_2

produced is equated to the Kreb Cycle enzymes, while the rest is proposed to be produced by malic enzyme. It has also been shown be Chalmers and Rowan (1971), that during these stages the concentration of pyruvate increases, which indicates the increased activity of malic enzyme. Pyuvate is also considered to play an important role in modulating the malate enzyme activity. The NADPH released by malic enzyme reactions is utilised by NADPH dehydrogenase, in plants, on the exterior of the mitochondria.

2.5.4.2 Malate dehydrogenase (E.C. 1.1.1.37)

Malate dehydrogenase is able to catalyse the reversible oxidation of malate to oxaloacetate with NAD^+ as a cofactor. The reaction mechanism are shown below.

$$\text{L-malic acid} + NAD^+ \xleftrightarrow{\text{malate dehydrogenase}} \text{oxaloacetate} + NADPH$$

There are two forms of malate dehydrogenase in Eukaryotes. These include the malate dehydrogenase for the citric acid cycle, which operates within the mitochondria, and the other malate dehydrogenase can be found in the cytosol where it participates in the malate aspartate shuttle (Figure 2.6), (Nicholls and Fergusson, 1992). The shuttle is useful in exchanging reducing equivalents across the mitochondrial membrane in the form of malate/oxaloacetate, instead of NAD^+/NADH.

Weinger and Banazak, (1978) evaluated the crystallographic structure for pig heart malic enzyme, it was found that the enzyme has a molecular weight of 74000 Daltons and is a dimeric molecule of identical subunits.

In eukaryotic cells, malate dehydrogenase is believed to exist as a complex with fumerase and citrate synthase. Beekmans and Kanarek, (1981), showed that this is possible because the standard free energy for the malate dehydrogenase reaction is unfavourable for operation within the citric acid cycle.

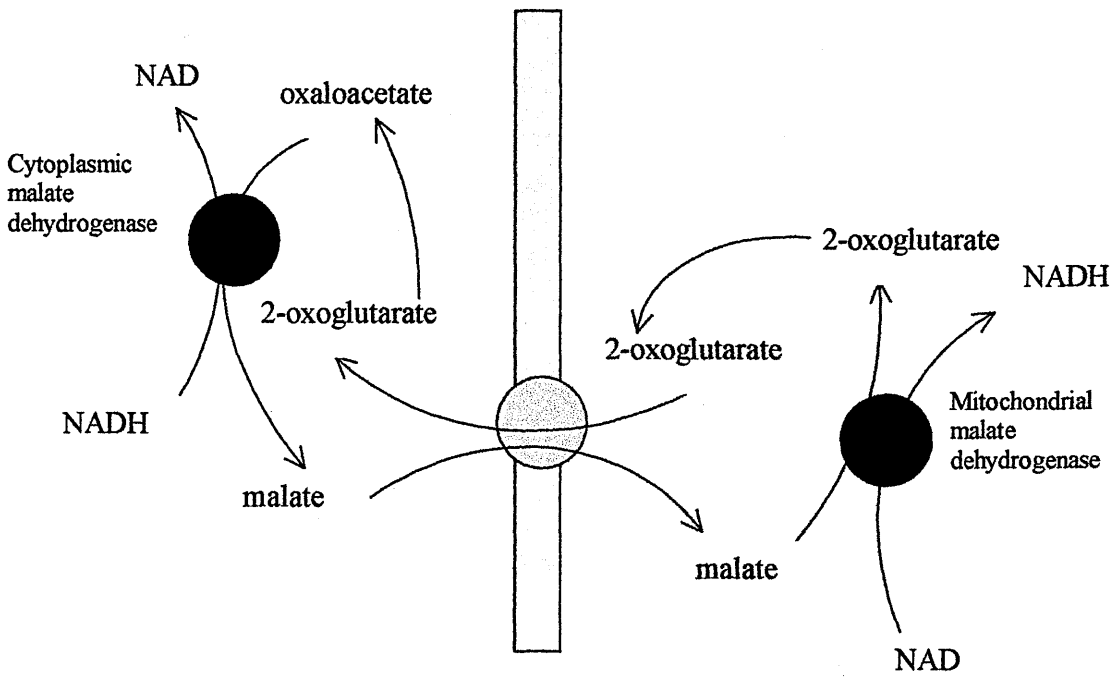


Figure 2.6 Shows the involvement of malate dehydrogenase in the malate/ aspartate shuttle

It is therefore believed that the formation of a citric cycle enzyme complex drives the cycle in the direction of oxaloacetate formation by facilitating the transfer of oxaloacetate from malate dehydrogenase to citrate synthase. It has also been shown that malate dehydrogenase has been able to form a complex with the mitochondrial

form of aspartate amino transferase, in order to facilitate the operation of the malate aspartate shuttle.

Malate dehydrogenase is also known to be allosterically regulated and high concentrations of malate can stimulate the production of oxaloacetate and inhibit the enzyme (Fahein et al 1988). It has also been shown that the presence of citrate can stimulate the formation of oxaloacetate. This is possible because citric acid has a similar chemical structure to malic acid, and it has also been shown that citric acid, malic acid and oxaloacetate bind to the same allosteric site, and this can lead to inhibition problems (Mullinax *et al.*, 1982).

Structural studies of malate dehydrogenase have shown that malate dehydrogenase from pig heart has no disulphide bridges. Between 30% and 40% of malate dehydrogenase is helical, and two molecules of the reduced co-enzyme can bind to the enzyme at any one time (Thorne and Kaplan, 1963).

3 LITERATURE REVIEW OF HORTICULTURE PRODUCE QUALITY

3.1 Definition and Classification of Food Industry problems, in terms of human perception of food taste

Humans perceive four principal tastes on the tongue: sweetness, sourness (acidity), saltiness and bitterness. Individual taste 'buds' on the tongue cannot sense all tastes, usually being specific to one. A fifth 'taste' known by the Japanese word 'umami' and often translated as 'meatiness' is often described. This is a subtle effect making the mouth feel full or the tongue larger but is caused chemically rather than physically and is particularly associated with condiments containing monosodium glutamate such as soy sauce.

Stevens, (1985) classified fruit and vegetables into two groups. The first are those in which the identifying flavour is produced by one compound or group of compounds e.g. sulphides in onions (Allium *cepa)*, or L-methoxy-3-isobutyl pyrazine in bell peppers (Capsicum annuum). The second are those which flavour is defined by a group of volatiles, none of which individually have an aroma characteristic of the vegetable, e.g. tomatoes. Even when there is a dominant flavour compound, the secondary flavours may also be important. For instance, there is considerable consumer interest in mild, sweet onions, characterised by high sugar content.

Solms, (1969) proposed an integrated flavour picture, starting with a base of nonvolatile compounds (such as sugars, acids, proteins and some amino acids), which give the fundamental taste and tactile effects. Another group of non-volatiles called potentiators and synergists that enhance the basic components supplement these. These would appear to include L-glutamic acid. Finally, there are the top-notes of volatile compounds that add the aromatic distinctiveness to a flavour.

The perception of a given flavour may change with the individual consumer. For example there are gender differences and age related differentiation in the strength of perception of taste. Isomeric forms of compounds such as sugars and amino acids taste different. For instance while L-glutamic acid has the unique 'flavour' mentioned above, its dextro form is almost tasteless. L-isoleucine is bitter whilst D-isoleucine is sweet (Beidler, 1965, Solms, 1969). D-glucose is sweet but L-glucose has no sweetness and is slightly salty (Beidler, 1965). Some of the factors of taste are described in the following section.

3.1.1 Sweetness

Sweetness is related to the concentration of sugars. The main sugars found in fruit and vegetables are D-glucose (dextrose), D-fructose (D-laevulose) and sucrose. The later could be enzymatically cleaved to form glucose and fructose. In some cases the presence of the sugars is sufficient to justify extracting the sugar product on a commercial basis.

Carbohydrates are an important energy source in the mammalian diet, so sweetness is an important characteristic in food. Sweetness in fruit is also an evolutionary trick,

along with noticeable colouring, to encourage seed dispersal. Selective breeding has enhanced this. Carbohydrates are stored in the roots and tubers of biennial and perennial plants to help them over winter and regenerate the photosynthetic machinery in the spring. Starch is the main storage carbohydrate in plants. Chilling, either by frost in the field, or in cold storage can cause changes in carbohydrate levels within roots and tubers, particularly with respect to individual sugars such as glucose. Parsnips, for instance, taste sweeter if harvested after frost. In cold storage, levels of individual sugars, increase in potato tubers. Sweetness is not equal in all sugars. For the principal sugars, it has been established that fructose is 1. 8 times sweeter than sucrose but glucose has only 0. 74 sucrose equivalents of sweetness (Schallenberger & Birch, 1975).

Sugar concentrations can be estimated by refractometry. Unfortunately, this method measures soluble solids, of which sugars only account for a part. There is a correlation between actual sugars and soluble solids but is not the same for all crops. For instance Hobson & Kilby (1985a, 1985c), found the relationship between sugars and soluble solids in cherry tomatoes to be different to that for round tomatoes. There is a correlation between perceived sweetness and soluble solids (Malundo *el al.*,1995) in tomatoes, but a closer correlation is apparant between perceived sweetness and sucrose equivalents divided by titratable acidity (Baldwin *el al.*, 1998).

3.1.2 Saltiness

Saltiness (salinity) is related to the concentration of salts such as NaCl and KNO_3. A small amount of salt in food enhances other flavours. Because sodium chloride salt is widely used as a preservative, many people expect and indeed enjoy a high level of saltiness in their food. Saltiness encourages appetite and thirst. High sodium in the diet is associated with heart and circulatory diseases, and therefore the addition of sodium salt to food is discouraged. However, small amounts of NaCl are desirable. Salts can be measured in juice by measuring conductivity. Increased salinity in produce feed leads to increased conductivity and salt in fruit. In tomatoes, increased salinity leads to a reduction in yield caused by a combination of smaller and fewer fruits. However, the quality (% Class 1) increases (Adams, 1991) up to a certain point. The reduction in fruit size (fresh weight) leads to a related increase in concentrations of sugars and acids in the juice. Petersen *el al.*, (1998) found that there was no increase in perceived sweetness with increased salinity in tomatoes by a sensory panel unless the salt was NaCl rather than $CaNO_3$ or KNO_3. Balibrea *et al.*, (1997) took nutrient feed salinity to high levels (140mM NaCl) and found decreased levels of hexose, starch, malic acid and citric acid compared with 70mM NaCl and control samples. Tomatoes grown in high saline conditions become hard and take on a polygonal appearance.

3.1.3 Sourness (Acidity)

There are several words defining the taste of acidity in English e.g. sour, sharp, tart, which have subtly different meanings - a bitter acidity, a fresh acidity, an astringent acidity respectivally.

Sourness relates to the presence of acids and can be loosely equated to pH. Standard measure of acidity, pH, measures the disassociated hydrogen ions. This does not always correlate with actual acids present or perceived sourness due to buffering effects within tissues. Most of the acids in plant tissue are weak organic and amino acids, which do not disassociate into ions easily. Both malic and citric acids are partially neutralised by potassium (Hobson & Kilby, 1984). All amino acids have a basic unit as well as an acidic unit so their contribution to acidity may be neutralised and, as mentioned above, their flavours are not necessarily acidic.

In order to study the potential use of biosensors for the measurement of quality of horticultural produce a review of the literature of fruits and vegitables was conducted. The primary ones studied here are tomatoes, potatoes and apples. These represent different types of tissue from different types of plants, including a tuber, and fruit from a perennial plant and fruit from an annual plant.

3.2 Tomatoes

There are four distinct types of the cultivated tomato *(Lycopersicon esculentum):* round, plum (used for canning), beefsteak and cherry (ssp *cerasiforme).* All are bilocular except beefsteak, which is multilocular. Two growth patterns are found, determinate and indeterminate. Indeterminate plants keep on growing and flowering and have to be layered throughout the growing season. These are commonly grown in North European greenhouses. Determinate or bush types grow only to a definite number of flower trusses and are cultivated outdoors in sunny climates.

The tomato fruit consists of a pericarp of parenchymatous cells surrounding locules, which contain the seeds. Seeds are loosely attached to the placentae, which are initially attached to the central columnella. The pericarp and the locular contents (otherwise referred to by researchers as 'flesh' and 'pulp') make distinct contributions to the flavour of a tomato as shown by the differences in sugar and acid contents. The locular contents contain more acid and less sugar than the pericarp (Winsor *et al.*,1962a; Davies & Kempton, 1975; Ho & Hewitt, 1986).

Kader *et al.*, (1978a) separated the mature green (MG) stage into MG3 and MG4. MG3 has no red colour visible internally or externally while MG4 has no external red colour but it has started to appear internally around the columnella. If there are any air spaces or if the seeds are still so hard that they are cut instead of moving out of the way when the fruit is cut, then the fruit is immature. Since the signs of maturity are principally internal, it is easy for immature green fruit to be accidentally picked along with mature by inexperienced pickers. Breaker stage occurs with the first show of colour on the skin, a pink-orange tinge usually around the blossom end. Inside the flesh and contents also start showing orange colour as chlorophyll decreases and 0-carotene and xanthophylls increase. The 'jelly' starts liquefying as the pectin breaks down. At this stage fruit are picked within a week of expected sale e.g. United Kingdom nurseries picking for multiple retailers. The fruit are ripening but still firm enough to withstand the rigours of the transport chain. Kader *et al.*, (1977) noted that tomatoes picked at breaker were often marketed as 'vine-ripe' in the USA. Red ripeness is usually 7 days after breaking although it can be delayed by storage conditions after harvest such as controlled atmosphere storage with very low

oxygen levels. The flesh is red with the development of lycopene and the locular contents slide out easily. They are generally regarded as too easily damaged and unsuitable for transport when this ripe.

3.2.1 The constituents of ripe tomato fruit

Table 3.1 Constituents of a Tomato (Davies & Hobson, 1981)

Constituent	Percentage %
Dry matter	5-7
Total sugars	48
glucose	22
fructoes	25
sucrose	1
Alcohol insoluble solids	27
protein	8
Pectin substances	7
hemicellulose	4
cellulose	6
Minerals	8
Decarboxylic amino acids	2
lipids	2
malic acid	4
citric acid	9
Other acids	2
ascorbic acid	0.5
pigments	0.4
volatiles	0.1
amino acids vitamins and polyphenols	1

3.2.2 Carbohydrates

Total Soluble Solids (TSS) are 60-70% sugars in tomatoes (Davies & Hobson, 1981). Measuring TSS by refractometry provides a good estimate of sugar levels and is the standard method used for this analysis. Soluble solids and reducing sugars increase in proportion to each other (Stevens, 1972a). However, the correlation follows a different line for different tomato types, so refractometry cannot be used for comparison between varieties of different types. It is inversely proportional to fruit size: e.g. beefsteak 4.0 Brix, processing (plum) 4.5 (Stevens, 1985), cherry 6.0 or more (Hobson & Kilby, 1985). The Brix value is the sum of TSS and reducing sugars. Fruit yield is also inversely related to TSS because of finite dry matter accumulation.

The soluble carbohydrate content of tomatoes is nearly all reducing sugars. Two reducing sugars are found in cultivated tomatoes; glucose and fructose. Sucrose is found in significant concentrations only in cherry types, contributing to their extra sweetness. In other types sucrose can be 1% fresh weight at fruit set but declines to <0. 1% by table ripeness (Davies & Hobson 1981). Traces of myoinositol have been found (<0.02%) and occasionally other trace sugars such as raffinose in individual varieties (Davies & Hobson, 1981). The sugar content of fresh market varieties is higher than that of processing varieties (Hobson & Kilby, 1984).

Fructose and glucose both increase towards ripening. However, whilst glucose concentration starts off higher than fructose in immature fruit the ratio decreases until ripeness (Davies & Kempton, 1975). Fructose inhibits sucrose synthase from making sucrose from starch (Schaffer & Petreikov, 1997).

Controlled atmosphere storage has been reported to delayed sugar development in fruit harvested mature green but there was only a minor reduction in the end concentration at ripeness (Salunkhe & Wu, 1973). Picha, (1986) found the ratio fructose: glucose to be similar throughout ripening in two round varieties and one cherry but increasing in another cherry variety (which had low sugars throughout). At ripeness they occur in roughly equal concentrations, with maybe a little more fructose depending on variety. Fructose is perceived as sweeter than glucose and sucrose so contributes more to sensory perception of sweetness. Hanna, (1961) found that sugars remain constant past ripening in fruit left on the vine (processing varieties).

Fructose and glucose increase with the first colour change and are generally reported to be higher in the pericarp than the locules. Sugar levels increase with solar radiation, although in the United Kingdom we may be talking about the difference between 2% fresh weight in spring/autumn and 3% in summer. Bucheli *et al.*, (1999) have shown considerable differences in sugar content and flavour acceptance from the same varieties grown at different latitudes.

Kader *et al.*, (1977) and Picha, (1986) compared store-ripened tomatoes with vine ripened both found that premature picking led to a reduction in sugars at red ripeness. Bisogni & Armbruster, (1976) found sugars were lower in store-ripened fruit than those ripened on the vine, but that the effiect may depend on season or variety. McCollum & Skok, (1960), using C^{14} glucose applied to leaves, found glucose mainly moved into fruit in preference to vegetative parts, but preferentially into certain clusters. Glucose did not move into ripe fruit, even though it reached the

stems and peduncles of such fruit therefore the abscission layer (between pedicel and fruit calyx) becomes impervious to sugar as fruit matures. Thus there is no benefit to sugar accumulation to storing ripe fruit on the vine but premature picking may lead to a reduction in sugars. C^{14} was transported into organic constituents in the fruit until turning, increasing at turning (breaker) then dropped to negligible by ripeness.

3.2.3 Acidity

Acidity in tomatoes reaches a maximum with the first colour change (breaker) then decreases past ripeness (Hobson & Kilby, 1985a) unless kept in cold storage from mature green (Hall 1968). This appears to be down to an increase in citric acid since malic acid and amino acids drop at this point (Davies, 1966a, b, Hobson & Davies, 1981). Kader *el al.*, (1977) concluded that the changes in titratable acidity were varietal in tomatoes.

Ripe tomatoes tend to have a pH of 3.95 (Sapers *et al.*, 1977, Stevens, 1972b). A few samples in Sapers *et al.*, (1977) survey were found to have pH up to 5.2, which were attributed to exceptional local growing conditions or overripeness. There is not a good correlation with pH and any specific acid or titratable acidity Sapers *et al.*, (1978).

Since there is more acid (particularly citric) in the locular contents than the pericarp, smaller fruit, with their higher proportion of locular contents to pericarp, contain higher concentrations of acids over all. Davies & Winsor, (1969) found smaller fruited varieties had higher titratable acidity than larger fruit. Picha (1986) found higher concentrations of acids in cherry types than round. Sapers *et al.*, (1977) found

low pH in small fruit. Most wild species of *Lycopersicon* have small fruit, which are unpalatable to humans because of their high acidity not being balanced by high sugars, which has limited their value in breeding for flavour despite their value in breeding for disease resistance.

Titratable acidity is closely correlated with citric acid and with potassium concentration. Tomatoes have a weak acid/strong base buffer system. Fifty per cent of acidity is neutralised in normal fruit. Sapers *et al.*, (1977) observed that pH was reduced approximately linearly by the addition of citric acid to canned tomatoes but the size of the response was varible. There may be wide variations in free acid and potassium ions with only minor effects on pH (Hobson & Davies, 1971). An increase in K^+ leads to an increase in organic acids to maintain a constant pH but still an increase in sharpness. Stevens (1972a) found phosphorus (as phosphate) to be significantly involved in differential buffering. Thus, titratable acidity measurements give a better view of overall variation in acid concentration than pH. Reduced water content (increased salinity) caused by K' salts leads to reduced acidity per fruit but an increase in acid concentration in the juice (Adams, 1991).

High nitrogen nutrition can lead to an increase in acidity (including malic and citric acids concentrations) but results are variable depending on year. Meanwhile high phosphate levels lead to a decrease in acidity (including malic, citric and glutamic acids concentrations) (Davies & Winsor, 1967; Hobson & Davies 1971).

Sapers *et al.*, (1978) found that postharvest infections by certain fungi increase pH.

3.2.4 Organic acids

Malic and citric are the predominant organic acids in tomatoes. Acetic, formic and transaconitic acids have also been recorded (Carangal *et al.*, 1954). In immature fruit there is a higher concentration of malic acid than citric acid but as the fruit ripens, malic decreases and citric increases or remains level (Hobson & Davies, 1971; Goodenough & Thomas, 1980). The ratio of malic acid to citric acid is regarded as significant with regard to flavour acceptance by some researchers (Davies, 1966a; Hobson & Davies, 1971). This ratio decreases through the colour change of ripening fruit.

There is more citric acid in the locules than the pericarp (Carangal *el al.*, 1954). It has been claimed that there is little varietal difference in citric acid concentrations but much variance in malic acid. However a recent paper (Bucheli el *al.*, 1999) shows that citric acid is one factor that is only influenced by variety, whilst malic acid, glutamic acid and the sugars can all be influenced more by environment. Goodenough & Thomas (1980) saw no pattern to changes in citric acid concentration during natural ripening in six varieties, but when kept in controlled atmosphere storage keeping them green, there was a decline in all. Higher concentrations of citric than malic acid were found in round and cherry varieties of tomatoes (Picha, 1986). He also found malic acid was higher in room ripened (from Breaker -1 day) than vine-ripened for the cherry varieties but not for round varieties. There was no significant difference for citric acid at this stage. The greener the fruit was picked the more citric and less malic acid the round types had at ripeness. The levels were similar with less change in cherry types.

Sakiyama & Stevens, (1976) found that, up to breaker stage, acids increase whether the fruit is on or off the plant but citric acid increases more off the plant when field stored (possibly a reaction to increased heat). After breaker the two acids decreased on or off the vine and the proportion of the two acids contribution to total titratable acidity stayed level. Pearce *et al.*, (1992) noted that malic acid concentrations in immature fruit grown in a glasshouse (in NFT) were up to double those in similar fruit grown in controlled environment (CE) rooms. This despite being grown at a 25 °C Day 15 °C Night regime, which produced lower malic acid levels than a continuous 25 °C in the CE rooms.

3.2.5 Amino acids

Amino acids have not been studied as much as organic acids and sugars because their role in flavour is not so clear. Kader *et al.*, (1978b) stated that some amino acids contribute to taste while others have a buffering action that also affects taste.

Glutamic acid is the amino acid present in the highest concentration followed by aspartic acid (Carangal *et al.*, 1954; Freeman & Woodbridge, 1960; Davies, 1966b; Yu *et al.*, 1967; Kader *et al.*, 1977,1978a, b). Some researchers also detected γ-amino-butyric acid in as high concentrations as aspartic acid. Glutamic acid increases dramatically (up to 10 times) during the colour change (Freeman & Woodbridge, 1960; Davies, 1966a; Hobson & Davies, 1981); aspartic acid increases on a smaller scale. Serine, though present in only small concentrations, has been observed to increase to a maximum at the breaker stage and fall afterwards (Freeman & Woodbridge, 1960). Other amino acids show no clear trends during ripening but total

amino acids level drop slightly at the breaker stage. Amino acid concentration is slightly higher in locular tissue than pericarp (Davies & Hobson, 1981).

Carangal *et al.*, (1954) showed that increasing NO_3-N in proportion to potassium and phosphorus in the fertiliser led to increases in glutamic acid. However, if NH_4+-N was used as the nitrogen source instead, an increased proportion of nitrogen led to a large decrease in glutamic acid and therefore of total amino acids.

Freeman & Woodbridge, (1960) looked at the role of amino acids in relation to quality. They noted that premature harvest led to poor quality on ripening. Their analyses of amino acid content (individual and total) showed that storage ripened tomatoes from all stages had slightly higher amino acid content than vine ripened ones. For the major individual acids, glutamic and aspartic, however, this effect was much more pronounced, (leading to the suggestion that the break in the metabolic pathway when the tomato is picked prevents glutamic acid being metabolised in the normal way and thus affecting flavour).

It has been suggested that certain amino acids are precursors of volatile aroma constituents (Yu *et al.*, 1967, 1968a, b; Hobson & Davies, 1971). Both glutamic and aspartic acids increased with ripening (Freeman & Woodbridge, 1960, Yu *et al.*, 1967). Davies (1966a) confirmed the increase in both locular contents and pericarp. In that experiment, glutamic acid was recorded in higher concentrations than malic acid in whole fruit although not in separate tissues. Yu *et al.*, (1968b) found that alanine, aspartic acid, leucine and valine all converted to glutamic acid by internal enzymatic reaction during the mature green stage, but there were signs that the reaction varies with growing environment and variety.

Kader *et al.*, (1977, 1978b) tested the hypothesis that tomatoes picked too prematurely have higher levels of glutamic acid on ripening than vine-ripened fruit and this is related to the detection of an 'off' flavour in-sensory tests. They picked tomatoes at six different stages including immature green (>10 days from picking to breaker) and partially mature green (6-10 days picking to breaker) and ripened them in store. They found higher levels of glutamic acid in ripened tomatoes that had been picked before breaking than those picked at or after. This corresponded with the detection of the 'off flavour by a sensory panel. However, manipulating samples with monopotassium glutamate did not reproduce the flavour effect so they concluded that glutamic acid was not playing a direct role in 'off flavour. They also noted that fruit picked 'Light Pink' late in the season (October) ripened slowly and had an 'off flavour.

Winsor & Adams, (1976) noted 'off-flavour' in some tomatoes imported to the United Kingdom which did not relate to titratable acidity or sugars. They suggested the development of a rapid test to detect this flavour would be useful. Hobson & Kilby (1985b) found glutamic acid at 0.1-0.339/100 ml sap in six cherry tomato varieties and 0.11-0.13 g/100 ml in two round varieties (all glasshouse, rockwool grown). They noted that their sensory panel reacted negatively to cherry tomatoes with the highest glutamic acid content despite good sugar and titratable acid levels. Bucheli *et al.*, (1999) analysed tomatoes of a range of varieties grown at two separate sites at different latitudes. They found a negative correlation of glutamic acid with the required 'fruitiness intensity' flavour associated with a positive correlation for

sugars and organic acids in this flavour. There was more variation in glutamic acid due to environment factors than variety of tomatoes.

3.3 Potatoes

The potato *(Solanum tuberosum)* originates in the Andes of South America. The aerial parts of the plants are poisonous, like its relatives the nightshades, but the tubers have become the staple starch food of Europe and the Americas. The tubers come in many colours from white to black with yellows, pinks and purples in between. From the wild species a wide range of varieties have been bred, diverse in tuber size, shape, colour, texture and flavour. The white and yellow-fleshed varieties are preferred in Europe. Yellow flesh is genetically dominant over white flesh. The skins are white to brown with some pink (e.g. 'Desiree'). The tubers are classified for culinary use according to texture. Mealy or floury potatoes lose structure during cooking and are suitable for mashed potatoes or jacket potatoes where the potato is not required to hold shape. Waxy potatoes are more suitable for roasting since they hold their shape during cooking and don't mash well.

The tubers are used at both the immature (new potatoes) and mature (maincrop, old potatoes) stages. The visual distinction between the two stages of development is the skin, which is thin, flaky and easily rubbed off of new potatoes but complete and only removable by peeling with a knife in mature tubers. New potatoes have a higher sugar content than unrefrigerated maincrop. Thanks to the effects of the Gulf stream certain areas of Britain have mild springs and are therefore able to specialise in early crops including new potatoes. These include Cornwall, Jersey (Channel Islands) and

Pembroke (South West Wales). Primary exporters of new potatoes to Britain include Cyprus and Egypt.

Potatoes are propagated from disease-free tubers of the previous year's crop, called seed potatoes, which are allowed to sprout (chit) in warm, dry, dark conditions. Tubers being stored for food are liable to sprout too and are often sprayed with a sprout suppressant chemical, since tubers that have started sprouting are poisonous and sprouting causes the seed tuber to wither. The sprouted tubers are planted outdoors in soil and often covered in horticultural fleece or polythene to keep off frost and trap warmth in the soil thus encouraging early growth. As the top growth develops (and the covers are removed), soil is heaped up over the plants into ridges. Before harvest, all top growth above the ridges is burnt off to make it easier to lift the tubers mechanically.

New potatoes are eaten fresh during the spring and summer, but mature potatoes can be kept in cold storage for up to eight months over the winter until needed.

3.3.1 Carbohydrates

Sugars enter potato tubers as sucrose from photosynthesis and are stored as starch, sucrose, glucose and fructose in the following equilibrium (Burton, 1966):

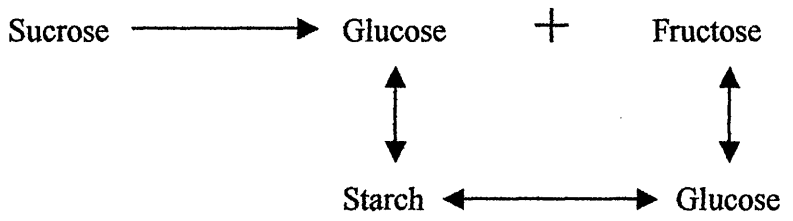


Figure 3.1 depicting the movement of sugars in potatoes.

Pritchard & Scanlon (1997) examined the variation in dry matter and sucrose, glucose and fructose levels in two varieties of potatoes compared with whole tubers. They suggested that, if only a section of potato is to be sampled, and then a section of the outer tissue from midway along the longitudinal axis gives the best approximation to the whole tuber.

3.3.2 Organic acids

There is enough citric acid in potato tubers for its extraction to have been considered worthwhile as a by-product of starch extraction in the 1940s. Burton, (1966) gives 2% of dry matter as normal with a range of 0.5-7%. Citric acid levels increase down the tuber (Burton, 1966). Minor organic acids found in potato tubers include malic, isocitric, oxalic, chlorogenic and sometimes pyruvic, (x-ketoglutaric and tartaric acids. Oxalic acid is associated with green areas of tubers and is poisonous. Chlorogenic acid is the cause of blackening of the flesh during cooking in some varieties. High citric acid levels can counteract high chlorogenic acid levels (Burton, 1966).

3.3.3 Amino acids

The amino acids present in the highest concentrations in potato tubers are asparagine and glutamine/glutamic acid. Asparagine is usually dominant but in some varieties glutamine is (Davies, 1977). There is strong variation in individual and total concentrations of amino acids between varieties and also between years and growing location. Changes also occur during storage (Burton, 1966; Davies, 1977).

Reducing sugars react with α-amino acid groups in the Maillard reaction during frying to produce a brown colour. Whilst levels of reducing sugars are the limiting factor in the degree of browning, some amino acids enhance the effect (Khanbari & Thompson, 1993).

3.4 Apples

The apple, *Malus Xdomestica (syri. Pyrus nialus),* has a long history as a cultivated fruit in Europe and has now spread through colonisation to be the most widely grown and eaten fruit world-wide. It is believed to have originated from wild Asian varieties carried and eaten along the trade routes. When the Romans arrived in Britain they had about twenty varieties, at least two of which are believed to be still in cultivation. Along with our native crab apple *(Malzispumila),* a small sour fruit eaten since prehistoric times whose high pectin content makes it popular for preserves, the apple has been cultivated almost continuously ever since in Britain. Early varieties appeared as seedlings and sports. The first record of a named variety in Britain occurred in 1205 in a legal document specifying an orchard of Pearmains. There are currently estimated to be 3000 varieties existent in Britain with about 300 in cultivation for market and about 7000 varieties from 4 genetic races worldwide. The variety 'Golden Delicious' accounts for 65% of the world market (Toussaint-Samat, 1992).

The principal areas of production are within 30 and 50' of latitude in both hemispheres. However, moderating effects, such as high elevation and proximity to

the sea, extend the range in places. Britain is on the northern edge of the growing area, but is helped by the warming influence of the Gulf Stream.

The varieties are split into three groups defined by their use: the cooking apples, cider and dessert (eating) apples. Cooking apples, dominated in popularity and production by 'Bramley's Seedling', are acidic in flavour, high in dry matter and unsuitable for eating raw. Although cider has historically been made with any locally available varieties, varieties such as 'Foxwhelp', which are typically high in tannins, are now accepted as an essential part of the blend.

Mangas *et al.*, (1998) looked at chemometric factors altering during ripening of six Spanish varieties of cider apple to see which ones were the most significant. They decided these were sugars (glucose, fructose, sucrose and sorbitol), which increase towards ripening, malic acid (citric acid was not studied) and the amino acids glycine, serine, valine and glutamic acid, which decrease throughout ripening (although asparagine was the dominant amino acid). Fructose was the most significant. Ripeness correlates with high sugars and pectins and low levels of malic and quinic acid, polyphenols and amino acids. In the final stages of fruit senescence, starch and malic acid metabolism along with the degradation of structural polysaccharides leads to increases in sugar and pectin concentrations. Quinic acid decreases probably in relation to phenolic acid syntheses. Amino acids levels in total drop.

3.4.1 Carbohydrates

Fructose is by far the dominant sugar, followed by sucrose then glucose. Sorbitol is also present in significant amounts.

The United Kingdom Nutrition Databank (UKND) published by the Royal Society of Chemistry gives the average sugar content of unsweetened apple juice as 2.6g glucose/100g, 6.3 g fructose/100 g and 1.1 g sucrose/100 g in 88 g water/100 g. In Elkins *et al's.*, survey (1996), glucose and fructose tended to similar levels but sucrose was often higher. In one year glucose levels averaged 2.46% (range 1.9-3.12%), fructose levels averaged 6.18 % (range 5.34-6.8%) and sucrose 1.32 % (0.56-2.54%). This survey included mixed variety samples and cider varieties.

Mangas *et al.*, (1999) studying cider varieties in Spain (not necessarily fully ripe) obtained figures of 1.34 ± 0.5 g glucose/100g, 4.45±0.94 fructose/100g and 1.88:1.07 g sucrose /100g (converted from g/kg). Wills et *al.*, (1998) noted total sugars as 11.7g/ 100g edible portion from Australian data.

3.4.2 Organic Acids

Akermann *et al.*, (1992) measured malic acid contents of apples by enzymatic kit methods and also by HPLC and was able to confirm Hulmes and Rhodes (1971) findings that 90% of the total acid content in apples was malic acid. They were able to show, that during development of the fruit malic acid content decreased. This was attributed to the mass dilution effect that may have been caused by the mass increase during the cell growth phase. After the apples had been left in storage, a further decrease in malic acid was seen and the possible cause of this was shown to be the

increased respiration which can lead to a decrease in malic acid, because it is a principal metabolic substrate.

Drake & Eisele, (1999) noted significant increases in citric acid levels towards ripening although citric acid is only present in small amounts compared with malic acid. Malic acid levels were over 50 times higher than those of citric acid in Drake & Eisele's study on the variety 'Gala' but averaged around 40 times higher in Elkins *et al.*, (1996) survey of a range of commercially produced apple concentrates. They were also highly variable (up to 100% CV) but variation seems to increase with low concentrations.

3.4.3 Amino acids

Elkins *et al.*, (1993) found asparagine to be the dominant amino acid in their survey of juice concentrates with an average of 41.1 mg /100 g (range 18.6-116.7 mg/100 g). Other significant amino acids were aspartic acid 13.9 mg/100 g (5.9-27.9 mg/100 g), glutamic acid 3.5 mg/100g (2.3-5.6 mg/100 g), serine 1.4 mg/100 g (0.81-2.2 mg/100 g) and alanine 1.3 mg/100 g (0.9-2.14 mg/100 g). All others recorded were present at less than 1 mg/100 g.

4 MEASUREMENT OF HORTICULTURE PRODUCE QUALITY BY CONVENTIONAL MEANS

Two major aims have been developed for this chapter:

This Chapter tries to address the need to measure horticulture produce quality. This is achieved by measuring taste of tomatoes via taste panel analysis. Key individual analytes in tomatoes are then measured using conventional methods and the data is subject to Principle Component Analysis (PCA).

The proposed biosensor is considered to be used for in field analysis. Whole horticulture samples e.g. potatoes cannot be tested with biosensors, or by conventional means. Therefore, a preparation step would be required. However, the preparation method must be cheap, simple, rapid, and amenable to decentralised use. This chapter tries to address this issue.

Methods:

4.1 Extraction methods

A selection of culinary tools was purchased to examine their efficacy at extracting liquid samples from fruit and vegetables, ideally without the use of mains power. The use of biosensors in 'the field' will require rapid and reproducible juice extraction techniques. The purchased tools were compared with the two standard laboratory extraction methods of freeze-thawing and homogenising in a high-powered blender.

4.1.1 Standard Laboratory Methods

4.1.1.1 Polytron® Blender

This is a high powered blender with an 8 mm diameter rotating head bearing a pair of oppositely placed blades in a cylindrical protective head. The blades are 4 mm deep and 4 mm wide set 25 mm apart. The blender is fixed vertically to a clamp stand. The blender head needs to be immersed in liquid to function efficiently, therefore samples with low juice content need to be roughly chopped and covered in water. Water of equal weight to the sample was added in these experiments. The sample is filtered or centrifuged to remove the solids. The blender both cuts and shears tissue and when used for a sufficient time (usually 10 - 20 seconds) will release sufficient amount of liquid sample.

4.1.1.2 Freeze-thawing

Samples are frozen in liquid nitrogen and stored in polythene bags at -20°C until required. They are then thawed at room temperature and the juice removed by pressing. This method softens the tissues and liberates more juice than other methods, since a large number of cells are broken open by the pressure of ice crystal formation. It is useful for dealing with large numbers of samples allowing analysis to take place at a convenient time. However, it requires at least 24 hours from sampling to analysis. It is tempting to use only the free liquid liberated at defrosting but this has higher sugar content than that held in the tissue and gives an unrealistic result. It is necessary therefore to press the thawed tissue and to thoroughly mix all the juices released.

4.1.2 Alternative Equipment for possible 'in field' use.

4.1.2.1 Presses

Various presses designed for mashing potato or crushing garlic were acquired from kitchen supply shops. Although different shapes and sizes, they have the same basic design. All are hand held presses consisting of two handles hinged together. At the end of the lower handle is a bowl with a perforated base whilst the top handle houses an integral pressure plate. The sample is placed in the bowl. When the two handles are squeezed together in one hand, the sample is pressurised by the plate and forced through the perforations in the bowl, liberating juice through the holes and around the pressure plate.

4.1.2.1.1 Garlic Press

The press comprises a bowl 12 mm deep at one end rising to 27 mm deep. It has a 2 mm thick base with 31 holes of 2.5 mm diameter in a 29 mm x 25 mm oval giving 5 holes/cm^2. The pressing plate is a 25 mm x 20 mm oval. The metal press is made in Italy, and sold by J Sainsbury plc.

4.1.2.1.2 Potato Ricer

The potato ricer (Lakeland Ltd, UK) is made of rigid polythene and is of similar construction to the garlic presses but larger, having a bowl of 70 mm depth and 80 mm diameter. The 76 mm diameter pressing plate is hinged to the upper handle instead of integrally moulded. The bowl is 80 mm diameter with interchangeable metal sieve plates. The plate recommended by the manufacturer for juice extraction has 7 concentric rings of 3 mm diameter holes (approx. 6 holes/cm^2). The others

have 3 rings of 4.5 mm holes (approx. 1 hole/cm^2) and 5 rings of 5 mm holes (approx. 5 holes/cm^2). The bowl has a bottomless liner, which was not used as it prevented contact between the plate bowl base and resulted in inefficient pressing the sample. There is a rest with three notches on the bowl to allow it to be balanced over a container.

This tool was useful for its capacity but the use of plastic in the design reduced the amount of pressure that could be applied to a sample compared with the metal garlic press. Since the sieve plate is loose it often sticks to the pressed sample and lifts out spilling solids when the pressing plate is lifted. The plates with larger holes let through tomato seeds and more pulped tissue, which then require filtering off.

4.1.2.2 Cutters

4.1.2.2.1 Multi-grater

This is a rotary grater marketed by Tefal® as the Multi-grater and purchased from Lakeland Ltd. It consists of a cylinder with a grating plate on the base. The cylinder is divided vertically by the holding point for the plate. A sample is put inside the cylinder and a lidded cylinder slides in above. As the lower cylinder is steadied with one hand, the top cylinder is rotated with the other. With a little pressure from the top cylinder the sample is forced down onto and across the grating plate causing slivers of tissue to be cut off and, sometimes, juice to be released.

The Multigrater was preferred, for ease of use, over the mouli. Grating was used as a preliminary to pressing

4.1.2.2.2 Zester

This is a stainless steel tool with a plastic handle designed to cut thin pieces of zest from citrus fruit. It was made in Italy and sold by J Sainsbury plc. It has a flat blade ending in five rings of 2mm diameter, which are sharpened internally to a cutting edge. Dragging the tool over a sample causes thin strips of tissue to be cut of release of juice. This devise is only of use on firm fleshed produce.

4.1.2.2.3 Knife

This is a small bladed kitchen knife with a pointed end, sharpened on both sides. The point of the blade is used to pierce the sample and twist as necessary to release juice from damaged cells.

4.2 Analytical Methods

The levels of analytes in produce tissues were analysed either chemically (Ninhydrin assay) for amino acids or using enzyme-linked methods for citric acid, L-malic acid, L-glutamic acid, D-glucose, D-fructose and sucrose. The results from these methods were compared with standard quality assessment methodologies, currently in use: - acid titration, pH and refractometry. Samples were also analysed by biosensors methods developed in this work.

4.2.1 Enzymatic Assays

Organic acids, sugars and L-glutamic acid were assayed using enzymatic methods with photometric detection. The assays were generally carried in cuvettes typically 3ml reagent per sample, in triplicate and read in a spectrophotometer.

4.2.1.1 Assays for sugars, organic acids and L-glutamic acid

Test kits manufactured by Boehringer Mannheim were used. In general the following format was used for all assays.

Reverse osmosis water was used in lieu of a sample as a 'blank' for the base line. Standards were used each time an analyte was measured to ensure the accuracy of the method.

A set volume of sample, standard or reverse osmosis water (used as blank in all tests) was pipetted into a 1 cm path length cuvette (Kartell PMMA 4.5ml standard cuvettes, UV grade) along with a fixed volume of buffer and other chemicals except the initiating enzyme. The cuvette was covered with Parafilm® and shaken gently to mix the reagents. The cuvettes were read in the spectrophotometer (Kontron Instruments Uvikon 930). Later after three minutes the cuvettes were again read by the spectrophotometer. This was done to detect any evidence of interferent species such as ascorbic acid. This reading was taken as A_1. The initiating enzyme was then added and the reagent mixed again as previously described. The time of incubation varied with each of the test kits (see individual assay details). When an endpoint had been reached it was called A_2.

The change in absorbance (AA) was calculated from the following equation:

$$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)blank$$

ΔA should be at least 0. 1 absorbance units to ensure accurate results

$$\text{Concentration} = \frac{(V \times MW)}{\varepsilon.d.v.1000} \times \Delta A \text{ (g/l)}$$

***Equation 1* general equation to determine the content of individual analytes such as glucose, malic acid and glutamic acid using commercially available test kits**

Where:

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of the substance to be assayed (g/M)

d = light path (cm)

ε = extinction coeffieient of end product at appropriate wavelength (l/mM/cm)

4.2.1.2 Titration

Titration measures the amount of alkali needed to neutralise an acid solution or vice versa. The assay can be carried out manually by adding alkali from a burette into an acid solution containing a pH indicator such as phenolphthalein. However, greater accuracy is achieved with an automatic titrator. The titrator is loaded with, in for example, sodium hydroxide. The sample to be titrated was first diluted as required. The titrator used was a Camlab Schott Gerate Titrator TR 154, operated according to manufactures instructions. Tomato juice samples are usually diluted 1 in 10 (5ml + 45ml water) and neutralised with 0.2 M NaOH. The settings settled upon for these experiments are: Min/100 % vol - 5 minutes, Delay - 50 seconds and Control - 2 pH units

4.2.1.3 PH meter

A Hanna Instruments HI 8521 digital pH meter was used to monitor pH according to manufacturer's instructions.

4.2.1.4 pH paper

Hobson and Kilby (1984, 1985) developed a quick test for tomato acidity, which gave a good correlation with titratable acidity. In the standards format, to 10 ml of sample, 190mg sodium phosphate was added. The resultant solution was then measured with a close range pH paper (BDH, pH 6-8).

4.2.2 Amino acids

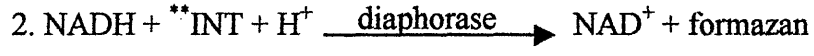
4.2.2.1 Ninhydrin Assay for free amino acids

This assay is derived from Moore & Stein, (1948) with the modifications of Singh *et al.*, (1978).

Test reagent: 150 ml glycerol, 0.625 g ninhydrin, 18.387 g citric acid, 133.3 µl of 150 mM $MnSO_4$, made up to 250ml in water. The test reagent (2 ml) was added to 0.1 ml of sample diluted 10-fold in water. The mixture was heated at 100°C for 12 min., diluted 2-fold in water and the optical density recorded at 570 nm. The method works for all amino acids except proline. The amount of total Amino acids was estimated by making a standared curve for L-leucine. The standareds used were 0 (distilled water), 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM of L-leucine. These standards may be stored frozen in aliquots until required.

4.2.2.2 L-glutamic acid assay

This colorimetric assay, based on Bergmeyer (1985), is available as a test kit (139092) from Boehringer-Mannheim. The reaction scheme is shown below.



*GLDH = glutamate dehydrogenase **INT = Iodonitrotetrazolium chloride.

The equilibrium of the first reaction is towards L-glutamate. By using up the NADH with INT, the equilibrium is moved to the right. The test kit contains four solutions whose content is shown below.

Solution 1 (Buffer)	Potassium phosphate (25mNI)/Triethanolamine (0.2M) + 13.2g/1 Triton X-1W® pH 8.6 (25 ml supplied in test kit) stable for 1 year at 4°C
Solution 2	35 mg lyophilisate consisting of 4U diaphorase + 28 mg β-NAD dissolved in 2.5 ml redistilled water. Thus diaphorase is approximately 16 U/ml. (Bergmeyer (1985) specifies 60 mg β-NAD as free acid in 12 ml redistilled water (6.7 mM), 9 mg diaphorase in 3 ml redistilled water (10 U/ml) Lyophilisate is stable at 4 °C for nine months. However when the sample is in solution then it is only stable for 1 week at 4 °C.

Solution 3	Iodonitrotetrazolium chloride (INT) solution (30 mg/50 ml redistilled water + 1.19 mM). 2.5 ml supplied. To be stored in the dark. Stable for 3 months at 4°C or 1 month at 20-25 °C.
Solution 4	Glutamate dehydrogenase (GLDH) solution. 1080 U/ 2.5ml glycerol (= 432U/ml). Stable at 4 °C. (Bergmeyer had specified 1200 U/ml in glycerol 50% v/v at pH 7.0).

When samples were not being tested then all solutions were stored at 4 °C. During the experimental procedure the solutions were brought up to 25 °C.

4.2.2.2.1 Method

The test was modified to allow measurement using reduced amount of reagents, thus enabling more samples to be assayed from one kit.

To each cuvette, the following sample additions were made:

Method	Kit instructions	Modified
Solution 1	600 µl	300 µl
Solution 2	200 µl	100 µl
Solution 3	200 µl	100 µl
Sample/Standard/Redistilled water (blank)	200 µl	100 µl
Redistilled water	1.8 ml	1.8 ml
Total (v)	3.0 ml	2.4 ml

Each one of the cuvettes were mixed, and the absorbance's were read at 492 nm. This was repeated after 3 minutes.

GLDH was added	30 µl	15 µl

Total volume (V)	3.030 ml	2.415 ml

The cuvettes were left to incubate in the dark for approximately 15 minutes and the absorbance's of blank and samples were read

4.2.2.2.2 Calculation

The calculation was done using equation 1 (as previously described).

For L-glutamic acid:

MW = 147.13

E = 19.9 $lmmol^{-1}cm^{-1}$(formazan at 492 nm)

Therefore:

$$\text{Concentration} = \frac{(2.415 \times 147.13)}{19.9 \times 1.0 \times 0.1 \times 1000} * \Delta A \text{ (*dilution factor)}$$

= 0.1785 * ΔA g L- glutamic acid/ l sample solution.

4.2.3 L-Malic Acid Assay by test kit

The test kit used was Boehringer-Mannheim L-malic acid test kit (No. 139068), based on the method described by Mollering (1985b). The following reaction scheme has been used in the test kits.

1. L-malate + NAD^+ $\xleftarrow{*MDH}$ Oxaloacetate + NADH + H^+

2. Oxaloacetate + L-glutamate $\xleftarrow{**GOT}$ L-aspartate + 2-oxoglutarate

*L-malate dehydrogenase and **glutamate oxaloacetate transaminase

The equilibrium in the first reaction is towards L-malate. By utilising the oxaloacetate with GOT the equilibrium is moved to the right. The increase in NADH is measured through the change in light absorbance at 340nm (alternatively 334 or 365nm). The test kit contained four solutions whose content is shown below.

Solution 1	440 mg L-glutamic acid in 30 ml glycylglycine buffer at pH approx. 10.0 (+ stabilizers).
Solution 2	210mg NAD lyophilisate dissolved in 6.0 ml redistilled water. Stable for 3 weeks at 4°C, 2 months at -20°C.
Solution 3	0.4ml glutamate-oxaloacetate transaminase (GOT) suspension containing approximately 160U (=400U/ml).
Solution 4	0.4ml L-malate dehydrogenase solution containing approximately 2400U (=6000U/ml)

4.2.3.1 Method

To each cuvette, the following sample additions were made:

Method	Kit instructions
Solution 1	1.000 ml
Solution 2	200 µl
Solution 3	10 µl
Sample/Standard/Redistilled water (blank)	100 µl
Redistilled water	900 µl

Mix each cuvette. Read absorbances in spectrophotometer at 340 nm after 3 minutes (A_1).

Solution 3	10 µl
Total volume (V)	2.220 ml

The samples were then mixed. Absorbance at 340 nm was measured after 10 minutes.

4.2.3.2 Calculation

For L-malic acid: MW = 134.09

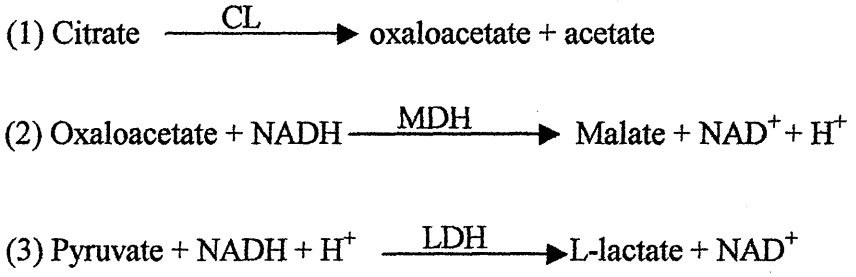
ε = 6.3 (NADH at 340nm)

Alternatively ε = 3.4 at 365nm or ε = 6.18 at 334nm

Thus using equation 1 the concentration of the sample is:

$$\text{Concentration} = \frac{(2.22 \times 134.09)}{6.3 \times 1.0 \times 0.1 \times 1000} * \Delta A \text{ (*dilution factor)}$$

4.2.4 Citric Acid Assay by test kit method



In the above reaction scheme citrate lyase (CL) was used to convert citric acid to oxaloacetate and acetate, while in the presence of the enzymes L-malate dehydrogenase was used to convert oxaloacetate to malate, and L-lactate dehydrogenase (LDH) was used to convert pyruvate, the decarboxylation product of oxaloacetate, into L-lactate. For both reaction schemes 2 and 3 NADH is oxidised to NAD^+. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

4.2.4.1 Method to determine L-citric acid:

The method was identical Boehringer-Mannheim L-citric acid test kit (Cat No. 0139 076). This approach was based on the work of Gruber and Mollering, (1966). A brief method is described below for information purposes.

1.4 g lyophilizate (solution 1) consisting of: glycylglycine buffer (pH 7.8), with MDH (136 U), LDH (280 U) and NADH (5 mg), was dissolved in 12 ml of RO water. Whilst, 0.3 ml of RO water, was used to dissolve 50 mg of citrate lyase (12 U, solution 2).

The solutions were added to the cuvette in the following order.

Method	Kit instructions
Solution 1	1.000 ml
Sample/Standard/Redistilled water (blank)	200 µl
Redistilled water	1.800 ml

The cuvettes were mixed and the absorbances were read at 340 nm after 5 minutes (A_1).

Solution 2	20 µl

The cuvettes were then mixed again and after 5 minutes the absorbances were read at 340 nm (A_2)

Equation 1 was used to calculate the concentration of the sample.

4.2.5 Sugars

Glucose and fructose were measured using the Boehringer-Mannheim test kit methods. The method described here is similer to the test kit method, but is included for information purposes. Total sugar content was estimated with the use of the refractrometer.

4.2.5.1 Refractometer

The refractometer used was a 0-50% sugar refractometer manufactured by Bellingham & Stanley Ltd, London.

A drop of liquid (minimum 3 µl) is dropped on the flat glass of the refractometer and covered with the end piece. By looking into the light via a red filter, through the refractometer, a reading can be obtained. An increase in reducing sugar levels leads

to an increase in the refractance causing the light area to move up the lens. The light area can be read off a scale in Brix (% total soluble solids w/w). Measurement of soluble solids gives a close approximation of total reducing sugars although they only make up a percentage of the total. However it gives no indication of which sugars are present in what proportions. There is a minimum detection point at 10 mg sugar/ml.

Hobson & Kilby (1985b) compared refractometer readings with sugar assays using the Technicon AutoAnalyser, which carried out a neocuproine assay on deproteinised sample. They found that the refractometer overestimated sugars by 40% in round tomato varieties but by only a third in cherry varieties. Therefore refractometry can only be used for comparisons of sugar levels within related genotypes.

4.2.5.2 Sugar assays by test kits

Boehringer Mannheim test kits for D-glucose (716251) and D-glucose/D -fructose (139106) were used.

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{Hexokinase (HK)}} \text{D-glucose-6-phosphate} + \text{ADP}$$

$$\text{D-fructose} + \text{ATP} \xrightarrow{\text{Hexokinase (HK)}} \text{D-fructose-6-phosphate} + \text{ADP}$$

$$\text{D-fructose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G-6-P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

$$\text{D-fructose-6-phosphate} + \text{NADP}^+ \xleftrightarrow{\text{PGI}} \text{D-glucose-6-phosphate}$$

Where:

PGI = phosphoglucose isomerase

G6P-DH = glucose-6-phosphate dehydrogenase

Reagents

Solution 1 5g powder consisting of, triethanolamine buffer at pH approx. 7.6, approx. 64mg NADP, approx. 160rng ATP, magnesium sulphate + stabilisers, dissolved in 27ml redistilled water.

Suspension 2 0.7ml suspension containing approx. 200 U hexokinase (=286 U/ml) + approx. 100 U G6PDH (=143U/ml)

Suspension 3 Approx. 0.7 ml suspension of approx. 490 U PGI (phosphoglucose isomerase) (=70 mU/ml) (for fructose).

Method

To each cuvette add, the following aditions were made.

Solution 1	1.000 ml
Sample/standard/water (=v)	100 µl
Redistilled water	1.900 ml

The sample was then mixed and the absorbances were read at 340 nm (alternatively 334 or 365 rim) after 3 minutes, the following additions were made:

Suspension 2 =	20 µl
V (for glucose) =	2.020 ml

Mix and incubate for 15 minutes. Read again to obtain A_2.

For fructose, then add:

Suspension 3	20 µl

V (for fructose) 2.040 ml

Mix and incubate for a further 15 minutes. This endpoint is A_3.

Calculation

Equation 1 was used to calculate the concentration of the sample.

4.3 Results and discussion

Efficient sample preparation is essential for subsequent biosensor analysis. The device will most likely be used in a field or pack house situation where laboratory extraction methods may not be practical due to the constraints imposed by decentralised analysis.

The following extraction methods were compared: freeze-thawing, blending, pressing whole material and grating followed by pressing. Extracts made by these methods had pH and soluble solids (Brix) measured, as well as the amount of juice produced. The ability of a zester and a sharp knife to extract juice, for refractometer tests was also investigated.

A variety of horticulture produce including aubergine, carrot, celery, Potato, and tomatoes of various varieties were examined by each extraction method. These samples were chosen to give a range of tissue types found in horticultural produce.

The presses were either used alone or following grating. The juice extraction efficacy was compared with two laboratory methods, freeze-thawing and the Polytron® homogeniser. Samples were cut into smaller pieces to fit into grater, press or Polytron blender. Freeze-thawing took place using large sections of fruit of vegetables except for tomatoes, which were cut in half for internal examination of

maturity but frozen as individual fruit. All extracts were strained through a coarse filter.

When using the Polytron® blender, all samples had an equal weight of re-distilled water added to facilitate maceration. The resulting pulp was strained through a coarse filter and gently squeezed out. With the samples such as mushroom or tomato, which formed fine soft particles, lower pressure was applied to prevent particles being forced through holes in the filter. This resulted in some juice being left in suspension. With harder tissue such as carrot, this was not a problem. The amount of juice extracted was calculated as the actual amount of liquid strained off at the end minus water added before maceration.

The efficacy and reproducibility of juice extraction were assessed as volume of juice extracted per 100g flesh weight, pH and soluble solids (Brix) using the Hanna Instruments digital pH meter HI8521 and Bellingham & Stanley 0-50% Refractometer, (Tables 4.1, 4.2 and 4.3). The extracts produced by the knife and zester, were very low volume and thus were only measured for soluble solids.

Table 4.1 Efficiency of juice extraction (ml of juice per 100 g fresh weight of tissue) for a range of horticulture produce. (n = 5 ± standard deviation)

Extraction Technique	Produce Type					
	Aubergine	Carrot	Celery	Mushroom	Potato	Tomato
Grated & Pressed	30±5	12±2	43±3	27±5	10±3	27±24
Pressed whole	28±3	<1	35±7	28±3	5±3	23±13
Polytron Blender	45±6	34±24	45±18	<1	23±14	64±6
Freeze/Thaw	63±13	52±6	47±7	54±4	44±4	54±10

Table 4.2 Total soluble solids. (Brix) determined by refractometry on juices extracted from a range of produce by different methods. Mean of 5 samples ± standard deviation).

Extraction Technique	Produce type					
	Aubergine	Carrot	Celery	Mushroom	Potato	Tomato
Grated & Pressed	4.5±0.5	6.8±0.45	3.0±0	5.7±0.84	5±0	5.6±1.34
Pressed	4.2±0.27	7±0	3.2±0.27	5.7±0.45	4.8±0.27	5.3±0.97
Knife	<1	<1	3	<1	4-5	<1
Zester	<1	<1	3.5	<1	4-5	<1
Polytron	2.1±0.22	3.8±0.45	2±0	3.1±0.22	1.9±0.22	5±0.94
Freeze-thaw	4.1±0.65	7.6±0.65	3.9±1.02	4+0	5.3±1.2	5.3±1.2

Table 4.3 pH of juices extracted from a range of produce by different methods (Means of 5 samples±standard deviation).

Extraction	Produce type					
Technique	Aubergine	Carrot	Celery	Mushroom	Potato	Tomato
Grated &	4.74	6.1	4.94	6.2	5.81	3.79
Pressed	±0.11	±0.08	±0.11	±0.06	±0.25	±0.09
Pressed	4.84	<1	5.11	6.32	5.42	3,85
whole	±0.07		±0.22	±0.05		±0.11
Polytron	4.75	5.71	5.25	6.29	5.42	3.77
Blender	±0.04	±0.11	±0.15	±0.03	±0.15	±0.05
Freeze/	4.32	5.86	5.08	6.65	5.26	3.28
Thaw	±0.22	±0.08	±0.27	±0.04	±0.15	±0.11

Table 4.4 Concentration of total free amino acids (mM) measured using the ninhydrin method. The juices were extracted by grating and pressing and also by freeze-thawing (mean of 5 samples ± standard deviation).

Extraction	Produce type				
Technique	Aubergine	Carrot	Celery	Mushroom	Potato
Grated &	13.16	8.57	10.28	74.7	18.53
Pressed	±2.22	±3.29	±0.90	±5.25	±8.63
Freeze-thaw	12.2	9.07	11.0	104.0	23.76
	±2.0	±2.16	±44.48	±6.38	±5.29

Table 4.5 Concentration of L-malic acid (mM) measured using L-malic acid test kit. The juices were extracted from a range of produce by grating & pressing and also by freeze-thawing. (Mean of 5 samples ± standard deviation).

Extraction	Produce Type				
Technique	Aubergine	Carrot	Celery	Mushroom	Potato
Grated &	2.99	8.83	25.14	9.49	12.25
Pressed	±0.67		±0.84	±1.0	±2.08
Freeze-	4.6	8.55	24.1	9.55	11.92
thaw	±1.34		±8.97	±6.34	±3.01

Table 4.6 Amount of D-glucose (mM) measured using D-glucose test kit. The juices were extracted from a range of produce by grating & pressing and also by freeze-thawing. (Mean of 5 samples ± standard deviation).

Extraction	Produce Type				
Technique	Aubergine	Carrot	Celery	Mushroom	Potato
Grated &	68.57	42.91	42.21	<10	36.51
Pressed	±5.58		±7.68		±5.62
Freeze-thaw	56.37	63.0	36.54	<10	23.44
	±15.15		±18.41		±1.29

For all crops, freeze-thawing liberated the most juice since the greatest number of cells were fractured. Freeze-thawing is therefore the basis of comparison with other methods. Extraction using the Polytron blender, necessitating the addition of water to the samples in most cases, yielded questionable results regarding the amount of juice extracted since some tissues (e.g. potato, mushroom) evidently held an appreciable amount of water within the tissue. The addition of water also complicated the calculation of the dilution factor. This showed up clearly with the soluble solids readings but the pH readings remained similar to those produced by other extraction methods.

Hobson and Kilby (1985a, 1985b) compared a hand-operated device, the "Autochop" (consisting of a convoluted blade repeatedly pushed down onto the sample in a container) combined with additional maceration with a Polytron blender and also with freeze thawing to prepare tomato samples for analysis. They found that whilst there was good correlation between the analyses made after Autochop + Polytron maceration and freeze thawing, the Autochop alone did not liberate the same levels of sugar as the other methods.

Yellow Springs Instruments (YSI), who supply a glucose analyser used in the potato processing industry, recommend extracting potato juice using the Acme Juicerator following a protocol designed by Sowokinos (1978). This machine macerates tissue then spins juice from the pulp at 3600 rpm with optional use of cellulose filters. However the device still requires subsequent flushing with distilled water or buffer creating a solution of inexact dilution. It is then recommended that the sample is covered and left to stand at 4°C for one hour before analysis. This machine is not

readily available in the United Kingdom. (Ferns Nutrition Center, 1997, Open Chute Inc, 2000, YSI, 2000,).

The extracts prepared by freeze-thawing yielded more liquid sample then those prepared by grating and pressing, although the concentrations of acids were similar. However, there tends to be more variation between samples prepared by freeze-thaw extraction. With sugars, there appears to be a relationship between amount of juice extracted per gramme fresh weight and the concentration of glucose. Where less juice was extracted by grating and pressing than freeze thawing e.g. potatoes or aubergines, there also appeared to be a correspondingly higher concentration of glucose. When samples were freeze-thawed it was noted that there were two stages of juice release, the first released by gravity on defrosting and the second requiring pressure. The juice released first always had a higher concentration of sugars, whilst the pressed extract contained sugar at lower concentrations. It appears that rupturing the tissue by mechanical means is less efficient at rupturing cells and cell bodies then the freeze-thawing method.

This does not necessarily mean that grating and pressing is a less adequate method for producing juice for sugar analysis than freeze thawing. Since the selected analytes are related to flavour, grating and pressing may well give a better representation of what is actually tasted in the mouth.

For comparison, Gazzani *et al.* (1998), extracting juice by rough chopping, (peeling if appropriate), homogenising and centrifuging, obtained 46 ml liquid per 100 g from aubergine, 51 ml per 100 g from carrot, 55 ml per 100 g from celery, 39 ml per 100 g from potato, 55 ml per 100 g from tomato and 37 ml per 100 g from mushroom.

They found pH of 6.14 in aubergine juice, 6.83 from carrot, 6.32 from celery, 6.27 from potato, 4.31 from tomato and 6.13 from mushroom.

4.3.1 Aubergine (*Solanum melongena*)

In this experiment, aubergines produced similar amounts of juice (25-35 ml/100 g) from grating and pressing compared to approximately twice the amount from freeze thawing and 1.5 times the amount from the Polytron extraction. This appears to be related to the physical durability of aubergines, which allows them to be compressed without breaking. In preliminary experiments, however, grating and pressing produced as much juice as freeze thawing (not shown). Also in the preliminary experiments grating or macerating the tissue caused a lot of froth on the juice, which was easily strained off through a coarse filter. When freeze-thawed, aubergine was the only tissue that held all of its juice when thawed out. Acidity was similar from grating and pressing, pressing and Polytron samples but a little higher from freeze thaw samples. There were differences in sugar levels between extraction methods as measured by both refractometry and specific analysis for glucose. These were attributed to variation within the fruit.

4.3.2 Carrot (*Daucus carota*)

Although potato could be easily crushed in a garlic press, this was not the case with carrot. Cutting and tearing the tissue into small pieces by grating allowed the mass of tissue to be compressed and some juice extracted. More juice was extracted by fine dicing in the Polytron blender but freeze-thawing liberated over four times as much juice as grating and pressing in this instance. However, grating and pressing is

more likely to resemble the way in which juice is released in the mouth.

It was also noticed that the freeze-thaw extract was duller and browner in colour than other extracts. It has been observed that the colour of the juice extracted from the inner core of the carrot root is bright and orange whilst that from the outer tissue more represents the colour of the freeze-thaw extracts. There were also differences in the juiciness and amino acids content of these tissues as well.

4.3.3 Celery (*Apium graveolens*)

Grating and pressing released about the same amount of juice from celery as from freeze-thawing, whilst simple pressing released only a little less. There were low total soluble solids readings (2-3 Brix) from most samples but higher readings (up to 5.5) occurred in the freeze-thaw extracts. Glucose readings also varied more in the freeze-thaw extracts (20.8 - 58.1 mM) than the grated and pressed (30.5-49.6mM), although the means were similar. Keller & Matile (1989) recorded sugars in celery petioles (the part eaten) as 20mg/ml (110mM) of mannitol, 10-1.5mg/ml (55-83mM) of hexose (glucose and fructose) plus <3.3 mg/ml (10 mM) of sucrose.

There was also greater variation within the freeze-thaw samples than the grated and pressed for amino acids and L-malic acid although, once again, the mean values were similar

4.3.4 Potato (*Solanum tuberosum*)

The lowest amount of juice was released when the potato was simply pressed(23 ml), however there was a small increase in juice content when the potato was grated and pressed (27 ml). Polytron blending was sufficient to release as much juice as freeze

thawing from new potato. Potatoes are known to be prone to variations in sugar levels throughout the tuber, particularly "sugar ends" at the shoot end of the tuber when the plant has been subjected to, for instance, water stress.

4.3.5 Tomato (*Lycopersicon esculentum*)

Three varieties, one round, one cherry and one plum, were used so most of the variation in volume and Brix can be accounted for by the different types with differing dry matter contents and the high soluble solids of cherry types. There was less variation in acidity. Freeze thawing yielded up to twice as much juice as other methods, but the blender yielded a fraction more. Given the softness and juiciness of ripe tomatoes, one might have expected them to yield a lot more juice without freeze thawing.

4.3.6 Measurement of taste

This section is an examination of differences of taste components between different tomato types and different varieties within each type. Five varieties of distinct taste characteristics were examined. These are classic round (C), beefsteak (B), plum (P), mini-plums (MP) and varieties for marketing as whole trusses (T). Between three and eight different varieties were examined for each type (see table below). In association with the States of Jersey Department of Agriculture, the varieties/types were subjected to a taste-panel examination.

A table of means of the measured taste components for each type is shown in Table 4-7. Significant differences were established for each parameter. For instance, mini-

plum tomatoes had significantly higher sugar levels (glucose or fructose) than, plum tomatoes, which in turn were higher than classic, beefsteak or truss tomatoes (for

Table 4.7 Compositional analysis of five tomato varieties. Total soluble solids (overall sugar content) were measured using a refractometer, while values for individual sugars and other acids was measured by the test kit methods. Total amino acids were measured with the ninhydrin method.

	Tomato Variety					
Description	Classic	Beefsteak	Plum	Miniplum	Truss	Overall mean
Total soluble solids (Brix)	5.01	4.36	6.23	7.22	4.86	5.50
pH	4.36	4.50	4.45	4.53	4.50	4.45
Amino acids (mM)	22.2	24.1	23.3	31.8	23.6	24.2
L-glutamic acid (mM)	10.2	10.1	11.3	13.7	12.2	11.1
Citric acid (mM)	26.6	19.0	30.7	22.8	26.1	25.6
Malic acid (mM)	4.27	3.68	2.47	2.22	3.84	3.30
Glucose (mM)	78.3	74.9	106.9	131.9	88.1	93.5
Fructose (mM)	87.1	76.1	107.6	146.2	91.0	98.1

which no difference was detected between them). Plum tomatoes had significantly higher citric acid levels than classic or truss tomatoes, which in turn were higher than beefsteak or mini-plum fruit. Malic acid levels varied between the types however the malic acid levels were much lower than citric acid levels (13% of citric acid level on average). The differences in mean organic acid levels were not reflected in differences in mean pH's. The combined mean values of hexoses (glucose + fructose) and organic acids (citric acid and malic acid) correlates well with the total soluble solids (Brix) as determined by refractometry. The amino acid levels of mini-plums are significantly higher than the other varieties, which were similar to each other.

The next stage of the statistical analysis identified differences between varieties within a type. For instance, type C7 had significantly higher sugar levels then most (but not all) other classic varieties and beefsteak variety B6 has low citric acid levels while B2 has high sugar levels; plum variety P5 has high citric acid levels and P2 has high glucose levels (in-depth analysis of the results is discussed later in this section).

Clearly, therefore, there are differences in the levels of taste components between varieties within a type. These differences are economically important, as taste will affect demand, price and profitability. Biosensors therefore have a potential use to determine taste of fruit varieties, objectively, rapidly and at low cost.

Table 4.8 shows the coefficient of variation within a type for each selected taste component. Coefficient of variation (standard deviation/mean x 100) is a measure of variability as a proportion of the overall mean. For the measured analytes (glucose, fructose, citric acid, malic acid, amino acids and glutamic acid), the % Coefficient of variation (CV) varied between 8.4% and 30.7%. This degree of variability is quite high and is considered due to natural variation between fruit of the same variety.

Table 4.8 Analysis within variety: Coefficient of variation between six different varieties of tomatoes.

	Tomato Variety				
Description	Classic	Beefsteak	Plum	Miniplum	Truss
Total soluble solids (Brix)	6.4	7.2	6.4	7.8	7.1
pH	1.1	1.1	1.6	1.5	2.2
Amino acids (mM)	20.0	15.3	16.9	20.1	8.6
L-glutamic acid (mM)	20.6	19.2	15.0	30.7	25.8
Citric acid (mM)	19.4	14.4	15.7	15.5	13.2
Malic acid (mM)	21.1	24.1	16.5	23.4	22.0
Glucose (mM)	16.6	14.1	13.5	14.4	11.5
Fructose (mM)	13.7	13.0	14.5	13.3	8.4

*Coefficient of Variation = 100 x (Standard deviation/Mean)

4.3.6.1 PCA analysis to determine key analytes that effect taste

These data were subjected to Principal Component Analysis (PCA). The analysis work took place at Horticulture Research International ((HRI), Wellsbourne, UK). PCA is a valuable mathematical test to visualise and summarise a complex data set. When the data are reduced down to two dimensions, it was found that glucose, fructose and Brix data are highly correlated (Table 4.9). This is the first component (dimension) and it is tentatively described as 'sweetness' due to the correlation's with glucose and fructose. The second PCA component is due to the negative correlation between pH and citric acid and so could be described as acidity. The first component accounts for 50.9% of the variability while the second component accounts for 23.4%. The coefficients and correlations with individual constituents are included in Table 4-9.

The two dimensional representation of PCA can be seen in Figure 4-1. Tomato varieties can be seen to be 'clustered' in the PCA and the positions of these clusters differs for different types. For instance, the mini-plum varieties (MP3, MP4 and MP5) can be seen to cluster in the right-hand side of Figure 4-1, which are distinct from plum types, which largely found in the top right-hand quarter of the diagram. The classic, beefsteak and truss varieties occupy a distinct space but overlap with each other.

This PCA representation can be compared with taste panel results (table 4.10 and 4.11). The taste panel was conducted by presenting four or five tomato varieties

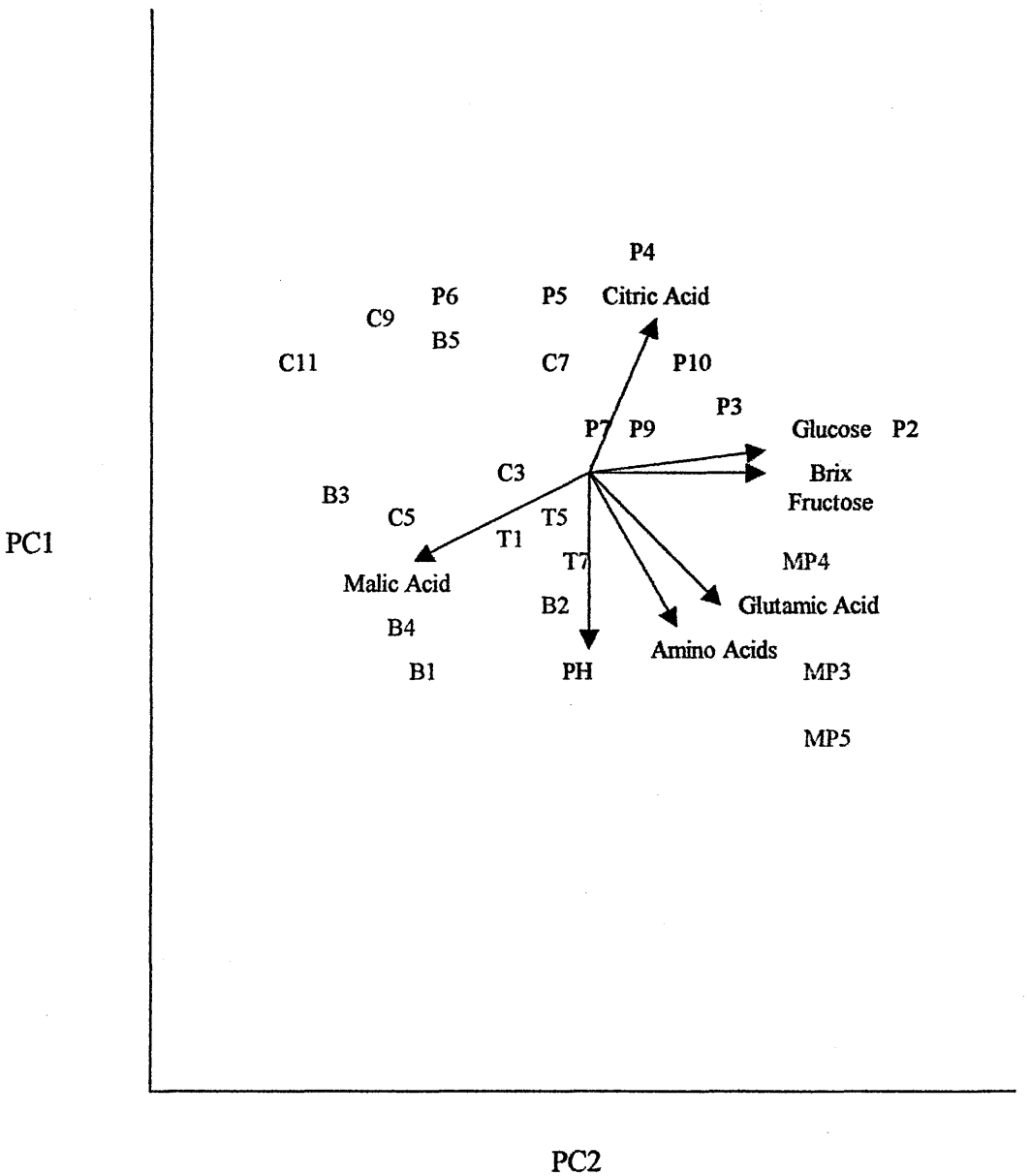


Figure 4.1 Principal component (PC) analysis of constituents of tomato varieties. Where, classic round = C, beefsteak = B, plum = P, mini-plums = MP and whole trusses = T.

Table 4.9 Coefficients and correlation with individual constituents of a Principal Components.

Measurement	Coefficient		Correlation	
	Component 1	Component 2	Component 1	Component 2
Brix	0.458	>0	0.925	0.041
pH	>0	-0.563	-0.190	-0.770
Amino acids	0.296	-0.476	0.597	-0.651
L-glutamic acid	0.348	-0.290	0.707	-0.397
Citric acid	0.179	0.568	0.036	0.777
L-malic acid	-0.318	-0.220	-0.642	-0.302
D-glucose	0.474	>0	0.956	0.002
D-fructose	0.464	0	0.937	0.010

Table 4.10 Tomato taste panal results for Classic round and Beefsteak varieties. The taste panel was conducted by presenting four or five tomato varieties within a type and asking each panellist to taste the tomato and give a preferences value from one to three. Where one donated to the tomato being tasty, two being 'average taste' and three donating to below average taste. The total number of taste panellists are also shown.

Classic round varieties				
Sample	One	Two	Three	total
C8	3	5	2	10
C2	7	1	3	11
C9	-	6	4	10
C10	1	1	2	4
C11	4	-	1	5
Beefsteak varieties				
B1	0	4	5	9
B2	-	3	-	3
B3	9	2	1	12
B4	5	1	5	11
B5	3	1	2	6
B6	-	1	2	3

Table 4.11 Tomato taste panal results for Classic round and Beefsteak varieties. The taste panel was conducted by presenting four or five tomato varieties within a type and asking each panellist to taste the tomato and give a preferences value from one to three. Where one donated to the tomato being tasty, two being 'average taste' and three donating to below average taste. The total number of taste panellists are also shown.

Truss Varieties				
Sample	First	Second	Third	Total
(C8)	2	6	4	12
T5	2	1	2	5
T8	3	1	1	5
T9	5	4	1	10
T10	3	4	5	12
Plum Varieties				
P10	4	2	2	8
P1	1	3	4	8
P2	5	5	2	12
P3	2	2	2	6
P4	4	3	4	11

within a type and asking panellists for first, second and third preferences. Forty-one different types were grown and 26 of these were analysed. The most preferred plum variety is P2, the co-ordinates of which are high on 'sweetness' but neutral on acidity. The beefsteak varieties are clustered within a fairly narrow band of sweetness (PC1) but a wider width of acidity (PC2). The most preferred beefsteak variety is B3, which has a typical sweetness value in PCA for beefsteaks but also is approximately neutral in the 'acidity' dimension.

4.4 Conclusion

4.4.1 Extraction

Initial results in this chapter show that freeze thawing ensures that the maximum amount of juice is liberated and is the standard extraction method where facilities and time allow (Table 4.1). Maceration in a high-powered blender followed by straining and filtering are useful and frequently recommended but are only justified if they can extract liquid from 'dry' samples since addition of water or buffer to the sample during the juice extraction process imparts an inexact dilution. In these experiments certain tissues such as mushroom or potato actually adsorbed added water in to the pulp (Section 4.3.4).

Simple pressing may release juice if the sample has a juicy, easily fractured structure such as tomato or citrus, preliminary 'rupturing'. This particularly applies to very rigid structures such as carrot but also to spongy structures such as aubergine. Even juice samples such as ripe tomato can benefit from a preliminary 'rupturing' of the structure. Grating performed well with such samples presumably because tissue

material is broken down into smaller pieces compared with the 'Autochop'. Therefore, a methodology-using grating followed by pressing is recommended for extracting juice from flesh produce outside the laboratory.

4.4.2 PCA analysis

Significant differences were observed in the levels of taste components between tomato type and between varieties within types. The components showing these significant differences are citric acid, amino acids, glucose and fructose (Table 4.7). However, a high degree of variation was observed between fruit of the same type so replicate measurements will be important in establishing significant differences in analyte concentration (Table 4.8).

These analyses show that standard analytical assay methods, and thus potentially biosensors can be employed to distinguish between varieties and types of horticulture produce on the basis of key taste components. Since the analytical data obtained is consistent with taste panel results, with regard to consumer preference, There appears to be a demand for tomatoes exhibiting a narrow range of citric acid levels (20-26 mM) whilst the sugar levels varied between different tomato types (Figure 4.1). It should be noted however that overall flavour of a product is a combination of the taste components and volatile flavour components, the levels of which may also vary between varieties.

5 L-MALIC ACID BIOSENSOR FOR HORTICULTURE PRODUCE MONITERING

5.1 Introduction

L-malic acid and citric acid are major organic acids in most fruits and vegetables. Organic acids contribute greatly to taste, particularly of fruit, with a balance of sugar and acid giving rise to a particular taste for specific produce (McGlasson *et al* 1998). Significant increases in L-malic acid concentration have been shown to serve as a primary indicator of fruit maturity (Davis 1966, Wang *et al* 1993). Hence measurement of L-malic acid provides a potentially more objective means of determining the ripeness and hence 'shelf life' of horticulture produce than simple appearance and taste.

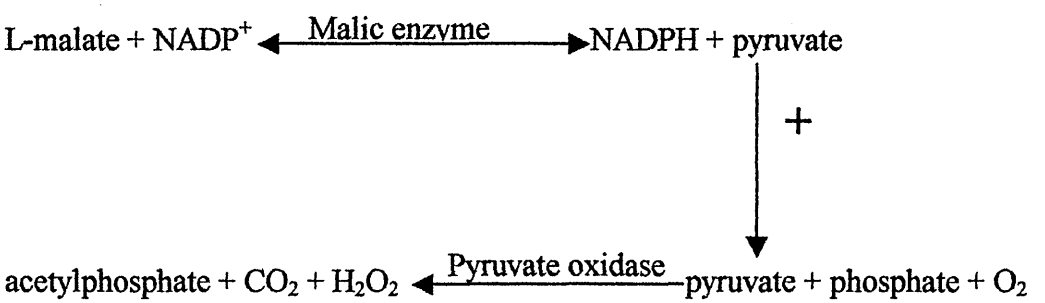
Current methods for the determination of L-malic acid in horticultural produce include both liquid and gas chromatography with the latter method being less widely employed due to difficulties in isolating and derivatising fruit acids (Jayaprakasha, and Sakariah 1998, and Barden *et al* 1997) Alternative methods include capillary isotachophoresis (Blanty *et al* 1996) and sequential injection-Fourier infra-red (FTIR) spectrometry (Schindler *et al* 1998) Commercially available test kits for L-malic acid also exist, based on enzymatic assay with photometric detection (Mollering *et al* 1989).

Biosensors, incorporating L-malic acid-specific enzymes coupled to electrochemical transducers have also been developed. Benefits include simplicity, rapidity,

economy, portability and minimal sample size. Two enzyme types have been used, malic enzyme (ME) (*Malate dehydrogenase (decarboxylating)(NADP)*), (EC 1.1.1.40) and malate dehydrogenase (MDH, EC 1.1.1.37), the latter catalysing the reaction:

$$\text{L-malic acid} + NAD^+ \overset{\text{MDH}}{\Leftrightarrow} \text{oxaloacetic acid} + NADH + H^+$$

Generally, the reduced NADH co-factor is measured amperometrically and related stoichiometrically to L-malic acid levels. Due to the low equilibrium constant ($k_{eq} = 6.4 \times 10^{-13}$ M), hydrazine, which reacts with the carbonyl group in oxaloacetate, has been used to promote the forward reaction (Almuiabad and Townshend, 1989). A similar approach using glutamate-oxaloacetate transaminase has also been reported (Chemnitius and Schmid 1989). An alternative approach uses an NADH oxidase (diaphorase) to regenerate NAD^+ with O_2 consumption measured with a Clark oxygen electrode (Maines *et al* 2000). Other strategies include the use of oxaloacetate decarboxylase and pyruvate oxidase to convert oxaloacetate to acetyl phosphate via pyruvate with O_2 consumption again measured with an oxygen electrode (Gajovic *et al* 2000).



The method described herein uses malic enzyme (ME), which has a k_{eq} of 5.1×10^{-2} M, thus negating the requirement for additional reagents to promote the forward reaction:

$$\text{L-malic acid} + NADP^+ \xrightarrow{ME} \text{pyruvic acid} + NADPH + CO_2 + H^+$$

Gajovic *et al* (1998) report the coupling of ME to salicylate hydroxylase, resulting in the regeneration of $NADP^+$ and L-malic acid determination via electrochemical measurement of oxygen consumption. Due to $NADP^+$ recycling, less then 0.025 mM of co-factor was required. Messia *et al* (1996) have used pyruvate oxidase in association with ME with amperometric determination of the hydrogen peroxide by-product at +650 mV vs. an Ag/AgCl reference electrode. Matsumoto *et al* (1996) recommend ME over MDH due to a preferred pH optimum of 7.8 compared with 9.5 for MDH.

The presence of electroactive interferents in real samples has led to the widespread coupling of mediators to enzymes in biosensor applications to allow the use of lower detection potentials (Tothill, 2001). An alternative approach uses electrocatalysts to selectively decrease the oxidation potential of the target analyte. At Cranfield, work has already been done in evaluating a large number of commercially available metallised carbons and identifying a screen-printable rhodinised carbon with excellent electrocatalytic and enzyme-immobilisation properties (Newman *et al* 1995, Tothill *et al* 1997, Kröger *et al* 1998a and Kröger *et al* 1998b.

5.1.1 Aim of the work

The aim of this work has been to develop stable, cheap, single use (disposable) screen-printed electrochemical sensors for the rapid and reproducible measurement

of L-malic acid in apple, potato and tomato horticultural preparations. Such an approach would provide a simple, rapid field-based tool for determining the optimum time to harvest produce with respect to the key parameters of ripeness and taste. Simplicity of the measurement process was paramount, due to the intended field-based usage of the device and target end-user. Consequently, electrocatalytic rhodinised-carbon was used in this study to allow the non-mediated detection of NADPH at low potentials – an approach that is of interest for the general exploitation of dehydrogenases in non-mediated biosensor applications. The performance of the device was compared against a standard photometric procedure and tested with real horticultural samples.

5.2 Materials and Methods

5.2.1 Reagents

Unless otherwise stated all fine chemicals were obtained from Sigma Chemical Company Ltd. (Poole, dorset, U.K).

Buffer: NaH_2PO_4/Na_2HPO_4, pH 7.4 (100 mM unless otherwise stated). Buffer-electrolyte solutions also contained 0.1M KCl. L-malic acid, L-tartic acid, L-citric acid, L-glutamic acid, NADP, and NADPH were from Sigma-Aldrich (Poole, Dorset, UK) and prepared in buffer-electrolyte as required. Malic enzyme (Malate dehydrogenase EC 1.1.1.40), from chicken liver (specific activity 19.6 units/mg) and hydroxyethyl cellulose (HEC) were purchased from Fluka Chemie AG (Buchs, Switzerland). Solutions were prepared in deionised reverse osmosis water (Elgastat System, Elga, High Wycombe, UK).

5.2.2 Sensor fabrication

Three-electrode devices were mass-manufactured in-house by a multi-stage screen-printing process using a DEK 248 machine (DEK, Weymouth, UK) and screens with appropriate stencil designs (60 per screen) fabricated by DEK Precision Screen Division. The stainless steel screen mesh was mounted at 45° to the print stroke with 77 wires cm^{-1} and emulsion thickness of 13 μm and 18 μm for the solvent and water-resistant screens respectively.

Devices were printed onto 250 μm thick polyester sheet (Cadillac Plastic Ltd., Swindon, UK). The circular electrocatalytic working electrode (planar area: 0.16 cm^2) was fabricated from MCA 4a (MCA Services, Cambs., UK), a commercially available carbon powder containing 5% rhodium plus promoters, made into a screen printable paste by mixing 1:4 in 2.5 % w/v HEC in buffer-electrolyte. The reference electrode ink contained 15% silver chloride in silver paste (MCA Services). The counter electrode and basal tracks were fabricated from I45R carbon ink (MCA Services). The basal tracks were insulated from the measurement solution using an epoxy-based protective coating ink 242-SB (Agmet ESL Ltd., Reading, UK). The electrodes were then heat treated at 125 °C for 2 hours, in order to cure the epoxy resin and to stabilise the electrocatalytic pad to allow prolonged use of the device in aqueous solutions.

Aliquots (10μl) containing varying quantities of ME in 10 mM buffer were pipetted onto the working electrodes, dried for >40 min. at room temperature and stored wrapped in silver foil at 4°C until required.

5.2.3 Electrochemical test procedure

Measurements were performed using an Autolab Bipotentiostat Electrochemical Analyser with GPES3 software (Ecochemie, Utrecht, NL). A 1.1 cm diameter Whatman 114 filter disc (Whatman Ltd., Maidstone, UK) was placed over the 3-electrode assembly which, when wetted with sample, completed the electrochemical circuit. Buffer electrolyte (40 µl) containing 10 mM of NADP, was deposited onto the filter paper and the working electrode poised at a potential of +300mV versus the Ag/AgCl reference. The amperometric measurement procedure was initiated and the electrochemical response was allowed to equilibrate for 240 s, after which 20 µl of sample solution was added to the filter paper. The change in response was recorded at 350 s (i.e. 110 s after sample addition). All test solutions were prepared in buffer-electrolyte and all tests performed at 34°C in accordance with the findings of Messia *et al* (1996). Since real samples contain appreciable levels of naturally electroactive species such as organic acids, the samples were tested simultaneously in the presence and absence of immobilised enzyme, using the Autolab in bipotentiostat mode. The difference in current between the two responses was taken as a measure of specific malic enzyme activity and provided a simple means of accounting for interference factors. All tests were performed in triplicate.

5.2.4 Test kit method

Sensor performance was compared against a standard L-malic acid test method based on a commercially available colorimetric malic enzyme test kit. The method is shown in chapter 4 (section 4.2.3).

5.2.5 Detection of optimum temperature for malic enzyme biosensor

The enzyme electrode was prepared as described in Section 5.2.2. Buffer electrolyte solutions containing 10 mM of NADP and L-malic acid (0.7 mM), were maintained at temperatures between 15 °C and 45 °C and measured in triplicate as described in Section 5.2.3.

5.2.6 Detection of optimum pH for the malic enzyme biosensor

Phosphate buffer ranging from pH 6 to pH 8.5 was used to determine the optimum pH of the enzyme biosensor. An increase in concentration of Na_2HPO_4 was used to make the buffer more alkaline while an increase in NaH_2PO_4 was used to make the solution acidic.

5.2.7 Determination of activity of malic enzyme biosensor over time

Aliquots (10μl) containing malic enzyme solution were pipetted onto two sets of working electrodes and air dried for one hour at ambient temperature. One set was then wrapped in silver foil and was stored at 4 °C while the other set was stored in a constant temperature room at 20 °C. L-malic acid (0.7 mM) was used to asses the biosensor activity according to method 5.2.3. The activity of the enzyme was checked at regular intervals during a period of 5 months.

5.2.8 Malic enzyme electrodes constructed using cellulose acetate membrane

The possibility of increasing the linear range for the malic enzyme biosensor was investigated, using cellulose acetate membranes. 10 μl of the enzyme solution was

added to the working electrode surface of the screen-printed electrode and the sample was dried for 40 minutes.

A 1% or 2% cellulose acetate (w/v) in acetone solution was prepared and the enzyme electrodes dip coated twice (1 minute intervals) in the cellulose acetate solution and left to dry for 45 minutes.

5.2.9 Determination of optimum detection potential for NADPH

The optimum detection potential for the oxidation of NADPH was determined by step-amperometry across the potential ranges -600 to +600 mV using potential and time increments of 100 mV and 200 s. Tests were performed on blank electrodes in glass beakers containing 10 ml of stirred electrolyte-buffer. Current responses were recorded immediately before each potential step in the presence or absence of 10 mM NADPH.

5.2.10 Tests on real samples

Preparation and testing of real samples using commercial test kit for L-malic acid is described in chapter 4. Samples were diluted in buffer (0.1 M phosphate buffer pH 7.4) as follows: Jonagold apple: ×100; Bramley apple: ×200; Russell Burbank potato: ×10; Mini plum tomato: ×10; and tested simultaneously using the biosensor and test kit method. The extraction method of grating and pressing was chosen, and was designed to be simple, rapid and easy to apply in the field. Three different potato and tomato samples and six different apple samples were analysed in triplicate.

5.2.11 Method for detecting L-malic acid by use of malate dehydrogenase

Two approaches were developed, one to detect L-malic acid in a beaker of solution and the other to measure L-malic acid by adding sample to the surface of the electrode pad.

For both experiments a stock solution of glycine/HCl buffer (pH 9) containing 100 mM of KCl was used. malate dehydrogenase (EC 1.1.1.37) enzyme powder (25000 U), (Biozyme, U.K.) was dissolved in 500µl of 0.1 M glycine buffer at a pH of 9. Glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1) solution (500 µl) was spun at 1300 rpm at 4 °C for 5 minutes in order to decant excess salts from the sample. The resultant pellet was resuspended in 500µl of glycine buffer.

L-Glutamic acid was also made up to a concentration of 5 mM in glycine buffer (10 ml). NAD^+ was also made up in concentrations of 5mM and 10mM in glycine buffer and the solution was kept at 4°C. A 10 mM stock solution of malic acid was also prepared in glycine buffer.

5.2.11.1 Detection of L-malic acid in stirred beaker

A 19.6 µl volume of malate dehydrogenase and 27.2 µl of glutamate oxaloacetate transaminase (GOT) were co-immobilised on to the working electrode surface. The electrodes were air dried for one hour, at ambient temperature.

Next 30 µl of Hydroxyethylcellulose (HEC) solution was prepared in 1.5% w/v water and pipetted onto the immobilised enzyme surface. The electrodes were then air dried for two hours. These electrodes were then stored in the fridge in silver foil.

L-glutamate (5 mM) and NAD^+ (5 mM) were prepared in 10 ml of glycine buffer containing 100 mM KCl. Enzyme-electrodes were then immersed in stirred glycine buffer solution. Aliquots of L-malic acid was added to the buffer solution at 100-second intervals. Resultant currents were measured at a potential of 300 mV against Ag/AgCl.

5.2.11.2 Detection of L-malic acid by droplet addition

A 19.6 µl volume of malate dehydrogenase and 27.2 µl of GOT were co-immobilised on to the working electrode surface. A circular 1.1-cm diameter Whatman 114 filter disc (Whatman Ltd., Maidstone, UK) was used to cover the three electrodes. A 20 µl volume of L-glutamic acid (5mM), and 20 µl NAD^+ (5mM) was then applied to the membrane-electrode-assembly. The amperometric response was measured at a potential of 300 mV against Ag/AgCl. A 20 µl volume of L-malic acid was added 200 seconds after initial sample addition and a reading taken after a further 200 seconds.

5.3 Results and Discussion

5.3.1 Determination of optimum Temperature for malic enzyme:

The activity of malic enzyme was examined at temperatures of 15 °C to 45 °C (Figure 5-1). It was seen that the maximum activity was evident at 35 °C. The enzyme exhibited 56 % of maximum activity at 15 °C compared with 71 % activity at 45 °C. Messia *et al.*, (1996) had demonstrated that the maximum activity of malic enzyme was at 34 °C. Since we had not tested at 34 °C, but our optimum temperature

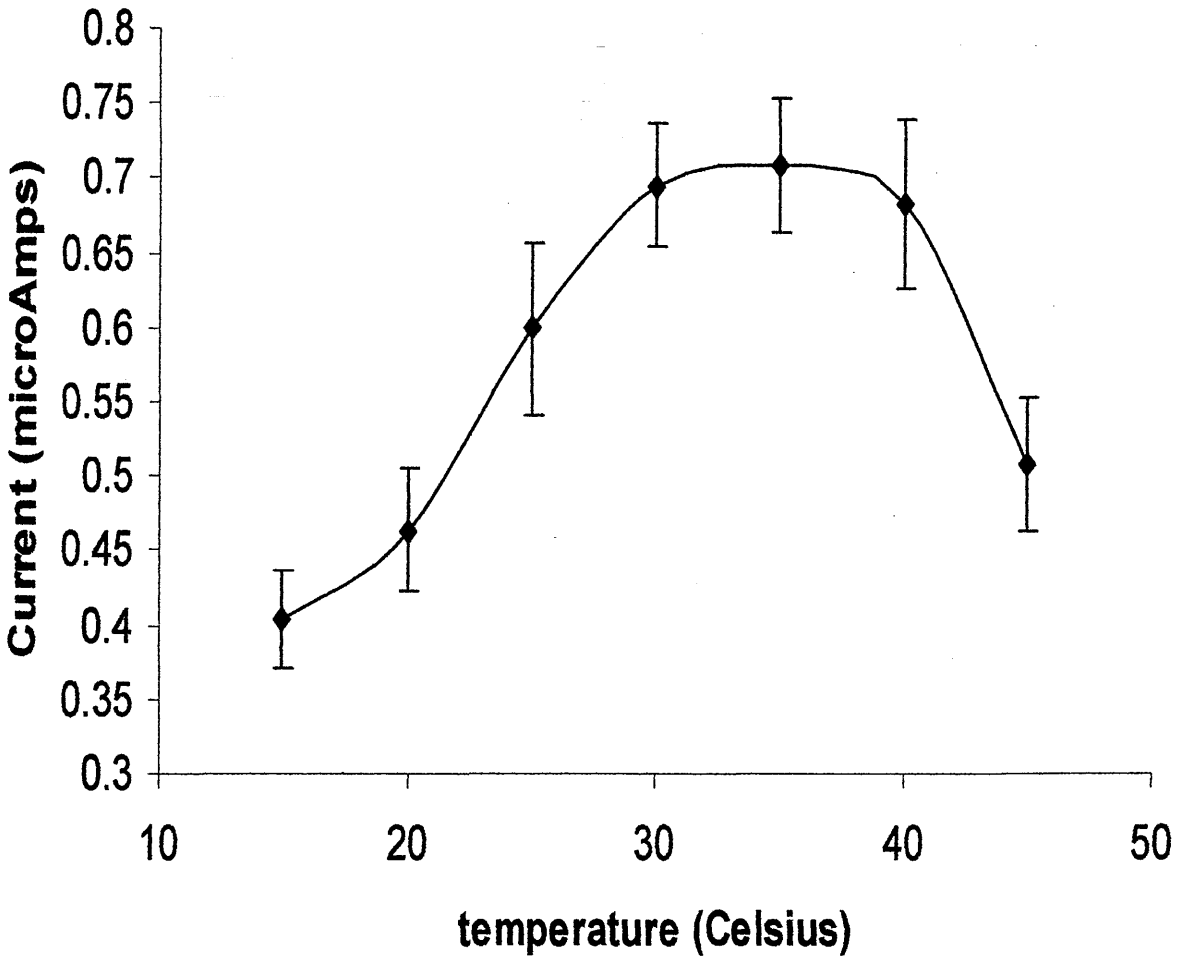


Figure 5.1 Activity verses temperature profile for malic enzyme. Each data point represents the mean of 3 individual measurements. The electrolyte buffer containing 0.7 mM of L-malic acid was heated in a constant temperature bath, at the required temperature. The working electrode was poised at a potential of 300 mV against Ag/AgCl. The amperometric measurement procedure was initiated and the electrochemical response was allowed to equilibrate for 240 s, after which 20 µl of sample solution was added to the application pad. The sensor response was recorded at 350 seconds. The net increase in current over the response recorded after 240 seconds was noted.

was evidently close to those values in literature, all further tested with malic enzyme were examined at 34 °C.

5.3.2 Determination of optimum pH of malic enzyme:

The optimum pH for enzyme activity was examined using phosphate buffer (0.1 M) across the pH range of 6 to 8.5 (Figure 5-2). The highest activity of malic enzyme was recorded at pH 7.8. The enzyme was able to retain over 90 % of maximum activity at pH 7.4 and this was the pH that was used in subsequent experiments. The activity decreased to 33 % at pH 6 while the activity was 81 % of the maximum value at pH 8.5.

5.3.3 Selection of optimum measurement conditions

A pH of 7.4 and a temperature of 34°C was used in all tests based on previous biosensor studies using ME (Gajovic *et al* 1998 and Messia *et al* 1996). The active site of malic enzyme is thought to be a sulfhydryl group, which can be stabilised using 20 μM 2-mercaptoethanol (2-MCE) (Matsumoto *et al* 1996). ME also requires trace amounts of divalent cations such as Mg^{2+} or Mn^{2+}. The ME stock solution used in this study contained 0.5 mM 2-MCE and 10 mM $MnCl_2$, which proved adequate for sensor performance even after buffer dilution (addition of 20 μM 2-MCE and 3 mM $MnCl_2$ to the buffer-electrolyte had no measurable effect upon sensor performance).

5.3.4 Choice of working electrode material and detection potential

The electrochemical behaviour of NADPH and a range of potential interferents with varying detection potential using both bare carbon and rhodinised carbon are shown in Figure 5-3 and 5-4. In all cases, increases in potential resulted in increased current responses. Highest responses were observed for L-ascorbic acid on both electrode types. However, rhodinised carbon proved superior for the oxidation of NADPH at applied potentials of +200 mV or greater compared with bare carbon. Using zero current as the baseline, the response ratios for L-ascorbic acid : NADPH : glutamic acid (the most responsive of the other organic acids tested) at potentials of +200 mV and +300 mV were 10.6:6.6:1.0 and 4.3:4.3:1.0 respectively on rhodinised carbon. On bare carbon, corresponding response ratios of 85.2:2.4:1.0 and 76.4:3.3:1.0 were recorded at the same respective potentials.

Although the rhodinised carbon (Figure 5-9) gave higher response values, the signal-to-noise (S/N) ratios obtained were similar to those obtained for the bare carbon electrodes. For both carbon types, the highest S/N values recorded (~120:1) for NADPH were at +300 mV. A maximum S/N value was apparent at the same potential for ascorbic acid, although the other acids showed an increase in S/N at the higher potentials. The data suggests no benefit in measuring at higher potentials since no improvement in the NADPH response relative to the selected interferents is apparent. Higher detection potentials increase the likelihood of the oxidation of other interferents. Rhodinised carbon, with a detection potential of +300 mV was used in subsequent studies.

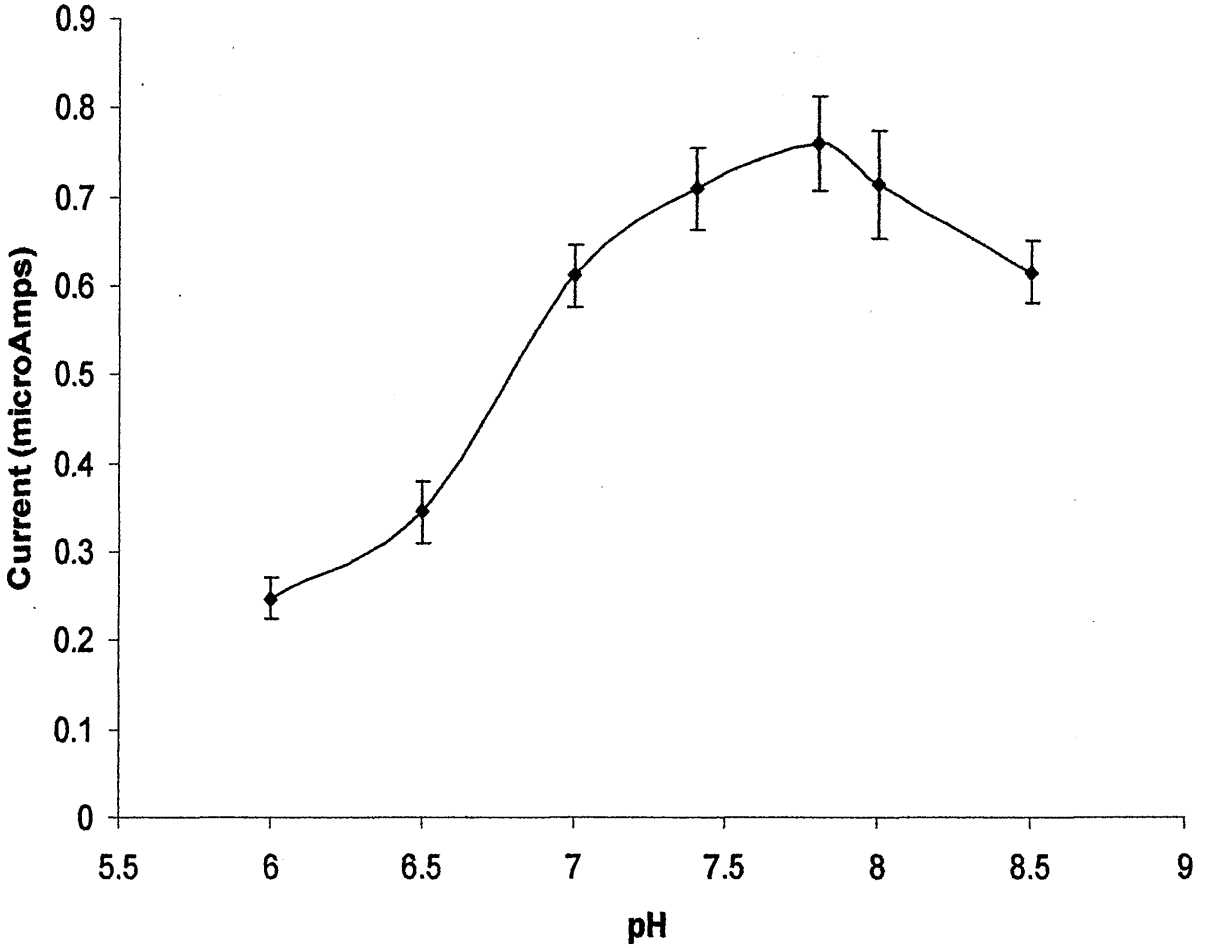


Figure 5.2 Activity versus pH profile for the L-malic acid biosensor. Phosphate buffer ranging from pH 6 to pH 8.5 was used to determine the optimum pH of the enzyme electrode. A 40 µl volume of electrolyte buffer (at the appropriate pH) was pipetted onto the applicator pad. The biosensor was left to equilibrate for 240 seconds prior to the addition of 20 µl of L-malic acid (0.7 mM). Current output was recorded at 350 seconds and the net increase in current over the response recorded after 240 seconds was recorded. The working electrode was poised at 300 mV against Ag/AgCl.

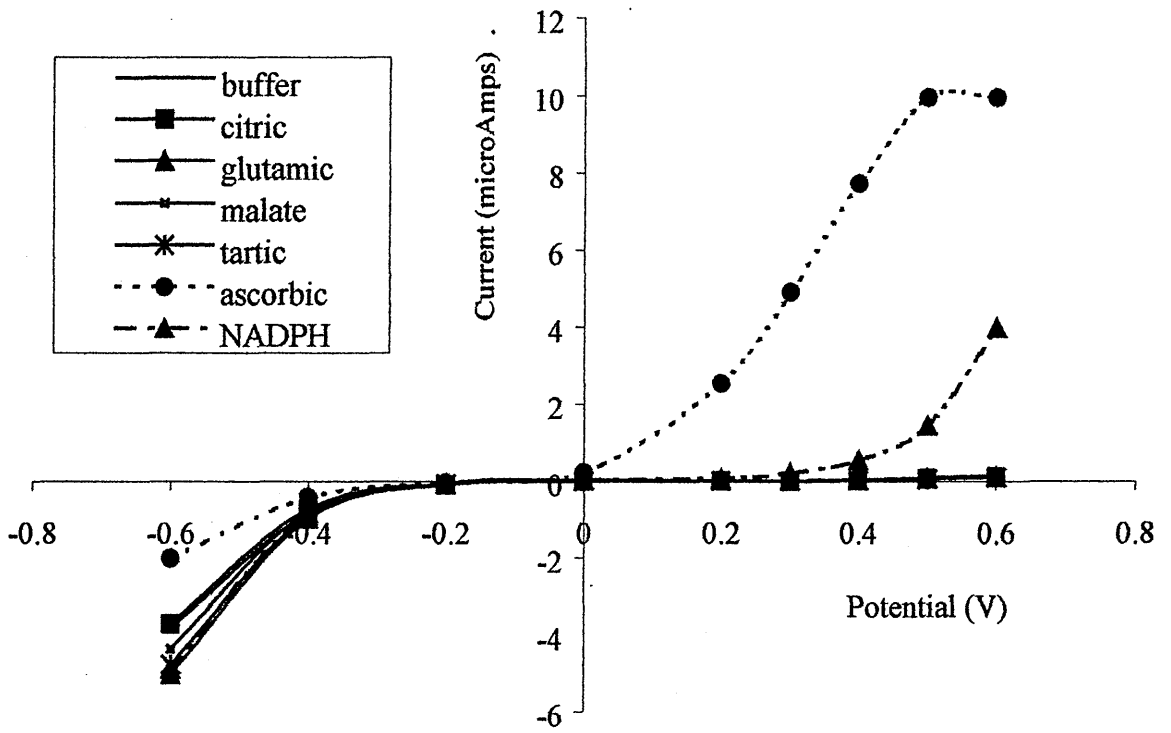


Figure 5.3 Current versus potential profile for NADPH co-factor and a range of possible organic acid interferents measured on bare carbon 10 mM preparations used with the mean of triplicate tests shown. The potential was maintained between -0.6 V to +0.6 V against Ag/AgCl. Potential and time increments of 100 mV and 200 s were used in the above experiment.

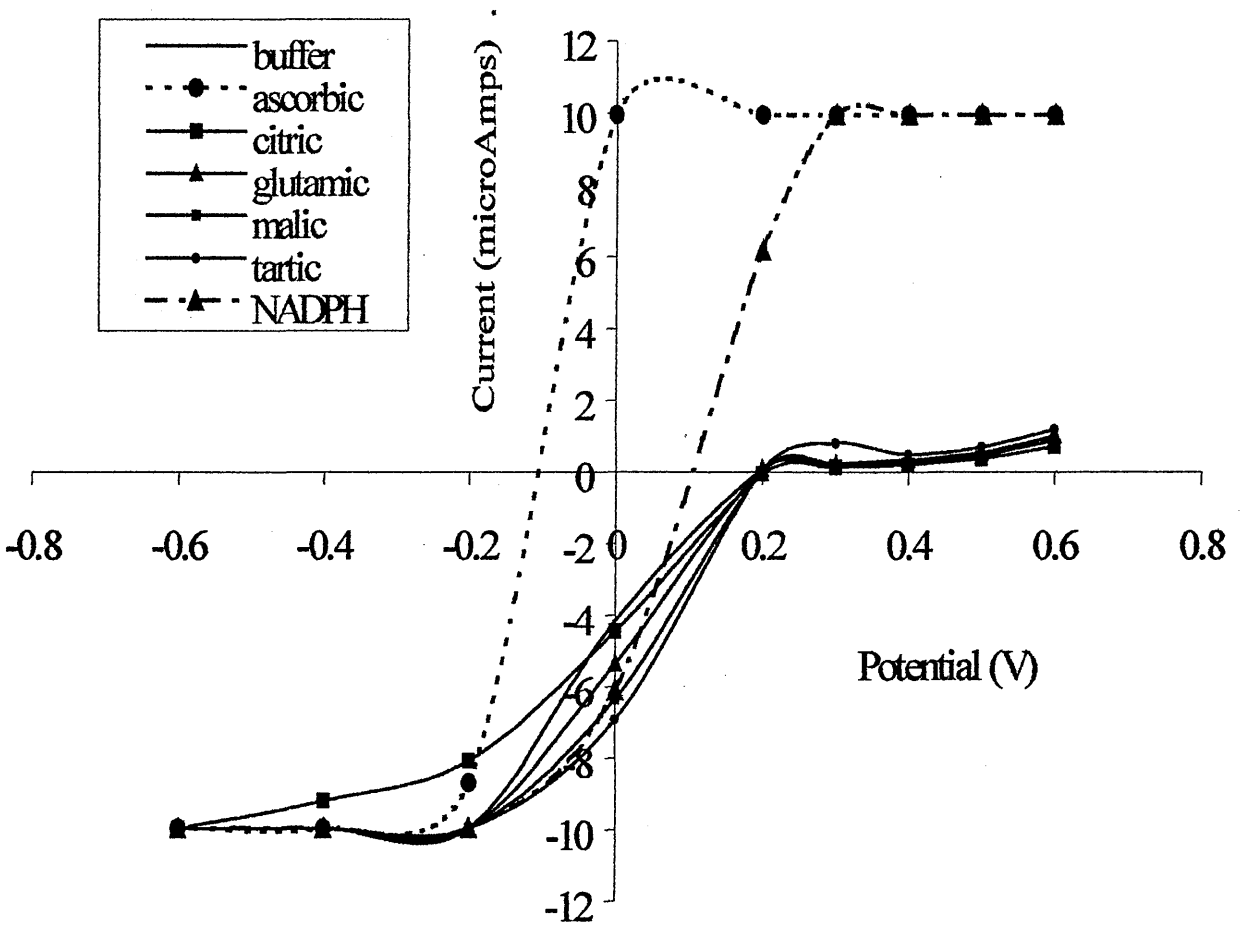


Figure 5.4 Current versus potential profile for NADPH co-factor and a range of possible organic acid interferents measured on rhodenised carbon 10 mM preparations used with the mean of triplicate tests shown. The potential was maintained between -0.6 V to +0.6 V against Ag/AgCl. Potential and time increments of 100 mV and 200 s were used in the above experiment.

Ascorbic acid oxidase could be used to convert the highly electroactive ascorbic acid to dihydroxoascorbic acid and water but would represent an additional preparation step with the removal of only one interferent species. In this study, the approach used was to subtract the background response of each sample using electrodes without enzyme from the equivalent enzyme electrode response. A final device would therefore incorporate two working electrodes – both rhodinised carbon, but only one dosed with enzyme –and a common reference and counter electrode. The difference between the enzyme-electrode and 'compensator' electrode responses would represent the L-malic acid specific response.

5.3.5 Optimisation of enzyme loading activity

Initial tests focused on determining the minimum enzyme activity required to generate a maximum current response from the system. In order to ensure that substrate concentration was not a limiting factor in the biosensor response, tests were performed under saturating levels of L-malic acid (Figure 5-5). A 2 mM L-malic acid solution was used, based on the criterion of saturation of $5 \times K_m$, where $K_m = 3.9 \times 10^{-4}$ M for malic enzyme from liver using L-malic acid as substrate.[23]

The amperometric response of sensors containing 0 – 0.6 mU malic enzyme per electrode to 2 mM L-malic acid in buffer-electrolyte were determined, with a maximum sensor response recorded at enzymes loading >0.35 mU/electrode. Accordingly, enzyme loadings of 0.38 mU/electrode were subsequently used to ensure an enzyme excess to maintain maximum biosensor using a minimal quantity of enzyme.

5.3.6 Sensor analytical performance

A linear relationship (Figure 5.6) between L-malic acid concentration (x) and current response (y) was observed up to 0.7 mM ($y = 9.24\times10^{-7}\ x + 5.77\times10^{-8}$; correlation coefficient r^2 value: 0.996). The limit of detection (LOD) for L-malic acid, calculated as 2.5 × the standard deviation (SD) of the zero analyte response was 0.028 mM.

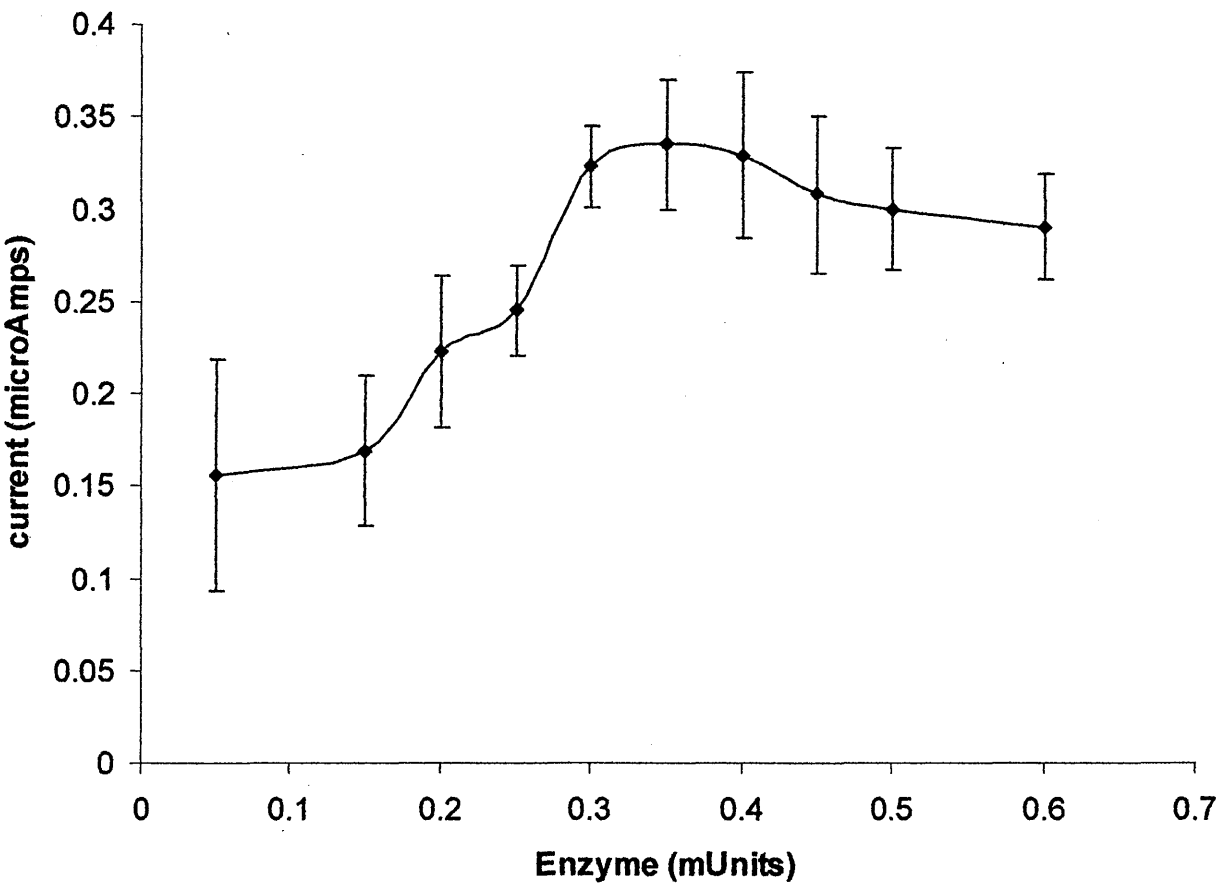


Figure 5.5 Effect of malic enzyme loading on the current response from L-malic acid biosensors

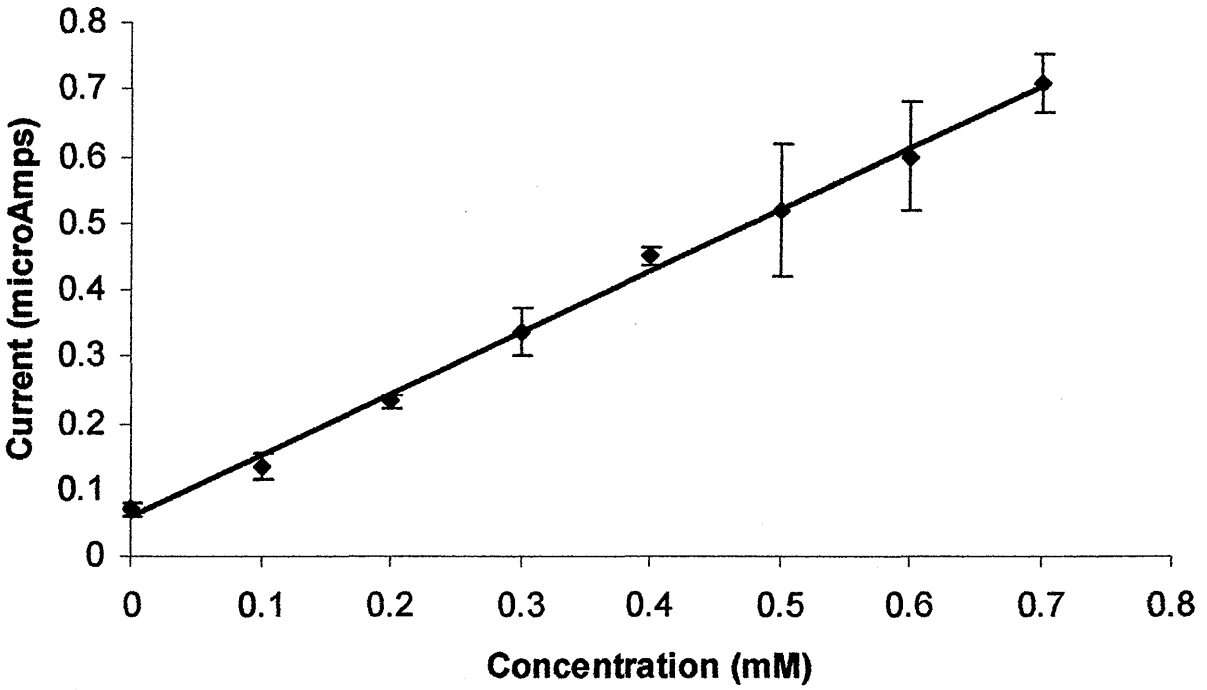


Figure 5.6 linear range of L-malic acid biosensor. A 40 µl volume of electrolyte buffer (pH 7.4) was pipetted onto the applicator pad. The biosensor was left to equilibrate for 240 seconds prior to the addition of 20 µl of L-malic acid. Current output was recorded at 350 seconds and the net increase in current over the response recorded after 240 seconds was recorded. The working electrode was poised at 300 mV against Ag/AgCl.

Thus, the linear dynamic range of the system was 0.028-0.7 mM. RSD values varied from 3.3-13.3% (6 concentrations, n=3). Across the range 0-1 mM L-malic acid, the data was best described by a polynomial relationship of $y = 6\times10^{-7}x^4 - 2\times10^{-6}x^3 + 2\times10^{-6}x^2 + 6\times10^{-7}x + 7\times10^{-8}$; correlation coefficient (r^2) value of 0.9990.

The sensor was also compared against a commercially available standard colourimetric malic acid test kit (Figure 5-7). A simple linear relationship ($y = 3.1332x$; $r^2 = 0.992$) was observed. The concentration range over which both methods can be directly compared is 0.028-0.7 mM, dictated by the linear range of the electrochemical method.

5.3.7 Activity of malic enzyme with time

Immobilised malic enzyme electrodes were tested with 0.7 mM of L-malic acid at temperatures of 4 °C and 20 °C (Figure 5-8). The enzyme was over 95 % active for three months when stored in the fridge. After 6 months the enzyme activity had decreased to 58%. On the other hand the malic enzyme electrodes were less stable when stored at room temperature where the enzyme maintained 95% activity for only 4 days. The activity nearly halved to 58% after 1 month and activity after 4 months storage was 4% of the maximum activity.

5.3.8 Inhibition effects

In addition to the electrochemical interferent effect (Figure 5-4), a number of compounds found in horticultural samples are also known to inhibit ME activity by competing with L-malic acid for enzyme binding sites. Since notable inhibitors include L-aspartic acid and L-citric acid, the same four organic acids as examined in

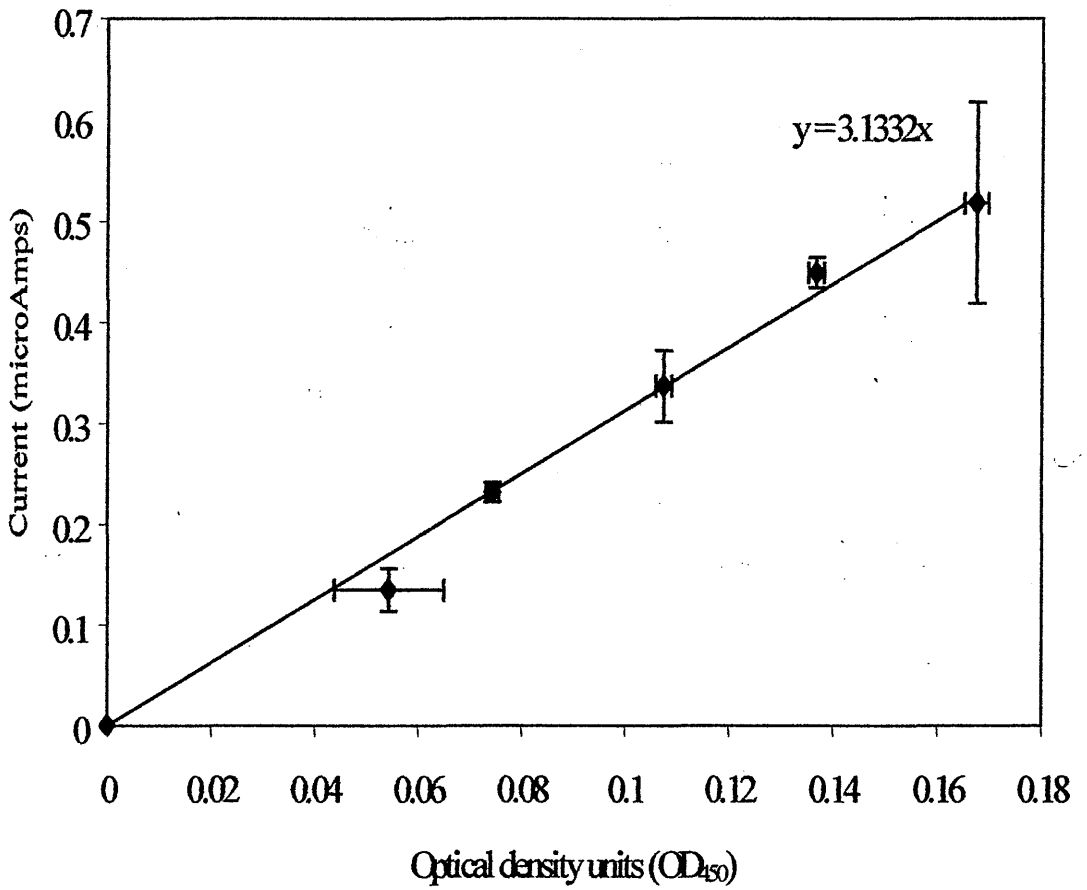


Figure 5.7 Correlation between electrochemically and photometrically determined L-malic acid concentrations. A 40 µl volume of electrolyte buffer (pH 7.4) was pipetted onto the applicator pad. The biosensor was left to equilibrate for 240 seconds prior to the addition of 20 µl of L-malic acid. Current output was recorded at 350 seconds and the net increase in current over the response recorded after 240 seconds was recorded. The working electrode was poised at 300 mV against Ag/AgCl. Background response values have been subtracted. Error bars = SD, n=3.

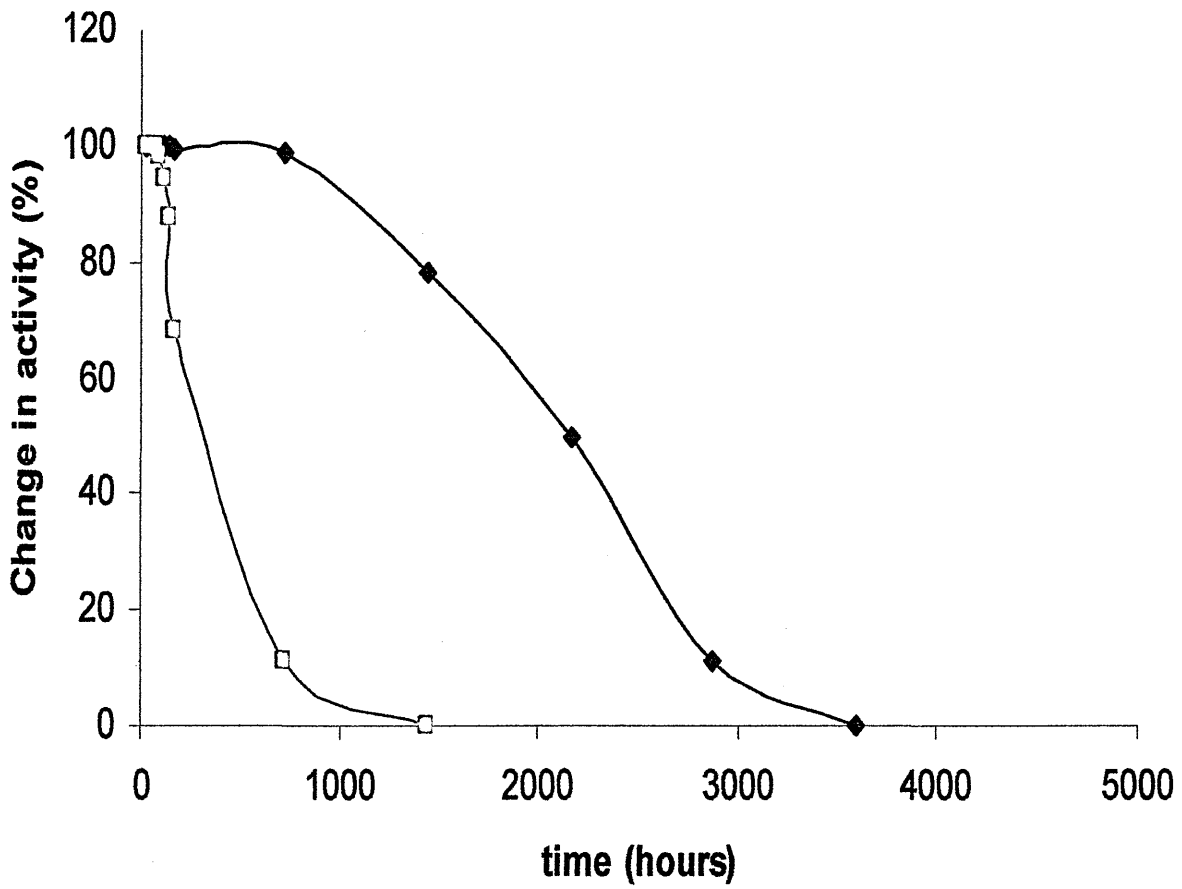


Figure 5.8 Activity of malic enzyme verses time profile for the malic enzyme biosensor. Each dot represents triplicate samples. Phosphate buffer pH 7.4 was used in all experimentations. Two sets of screen printed electrodes were stored at 4 °C (blue line) and 20 °C (pink line). The electrode was poised at 300 mV against Ag/AgCl.

the interference studies were evaluated with regard to their inhibitory effect on ME enzyme.

Firstly it was necessary to determine the extent of the sensor signal due to interference effects. The current response due to the direct electro-oxidation of 0.3 mM of the selected organic acids was determined on enzyme-free rhodinised carbon electrodes. Results are shown in Table 5-1, column 2. As previously indicated (Figure 5-4), L-ascorbic acid gave the highest response (197 nA) with all other acids, giving responses <75 nA.

Next, the response of the enzyme-electrode to each interferent organic acids was determined. No significant changes in sensor response were noted, indicating no reaction between the enzyme and these compounds (Table 5-1, column 3). Inhibitory effects were then determined by preparing solutions containing 0.3 mM L-malic acid and 0.3 mM organic acid interferent and determining the biosensor response (column 4). In all cases, an increase in sensor response was obtained due to the presence of L-malic acid substrate.

The difference between the enzyme-electrode and enzyme-free electrode response equated to the sensor response due to L-malic acid oxidation (column 5). Comparing the size of these responses to the L-malic acid response indicated no significant change in the enzyme-electrode performance (column 6). Thus it was concluded that inhibition of ME by other organic acids was not significant using the enzyme-electrode. Key results of malic acid specific responses being close to 100 % were able to show that use of an enzyme free electrode can be a simple and rapid approach in determining analyte concentration.

Table 5.1 L-malic acid biosensor inhibition studies. Full details of the method used to calculate malic enzyme inhibition by the selected organic acids are provided in text. Values are shown ± SD (n=3).

Interferent/ inhibitor	rhodinised carbon electrode response (nA)[a]	Enzyme-electrode response (nA)[a]	Enzyme-electrode response to Malic acid + interferent (nA)	Malic acid specific response (nA)[c]	Percent of malic acid specific response (%)[d]
L-Malic acid	68 ± 1.9	337 ± 35.1	350 ± 61.8[a]	282	100.0
L-Tartric acid	45 ± 4.2	62 ± 19.1	334 ± 39.4[b]	289	102.6
L-Glutamic acid	75 ± 15.2	53 ± 14.3	355 ± 71.2[b]	280	99.2
L-Citric acid	73 ± 13.0	68 ± 36.2	357 ± 42.8[b]	284	100.5
L-Ascorbic acid	197 ± 57.6	206± 6.6	474± 119[b]	277	98.2

[a] 0.3 mM of analyte

[b] 0.3 mM of analyte + 0.3 mM L-malic acid

[c] Difference between columns 2 and 4

[d] Calculated as: 100% × (column 5 response value/ column 5 response value for L-malic acid).

5.3.9 Testing the malic enzyme biosensor for the best type of real sample

Tables 5-2 and tables 5-3 were able to show a comparison of the CV values for the freeze thawed samples and grate-pressed samples. In the case of the biosensor the CV values were on average higher for the freeze thawed samples (23.75 %) as compared to the grate-pressed samples (13.63 %). The reverse was true for the test kit (5.49 % (freeze thawed) 9.79 % (grate pressed). It would therefore seem that in the case of the biosensor approach grate pressing liberates a more accurate analysis of L-malic acid. This method also has the advantage that it is less time consuming as compared to the freeze thawing method and also the grate pressing method can be performed in the field.

5.3.10 Testing real samples

A study by Hulmes and Rhodes (1971) has indicated that L-malic acid constitutes 90% of the total organic acid content in apples. Akermann *et al* (1992) have shown that an elevated amount of malic acid is present in immature fruits, including apples, which decreases as the fruit ripens. Measurement of L-malic acid therefore has possibilities as an objective indicator of fruit maturity. Correspondingly, the performance of the sensor in measuring L-malic acid levels in apple, potato and tomato preparations was determined and the results compared against the standard photometric test kit. The selected sample preparation method was chosen for ease of performance in the field. All samples were prepared freshly and tested simultaneously using the two methods.

Table 5.2 Results determined using L-malic acid biosensor, giving the mean malic acid concentration in mM, standard deviation (SD) data, and Coefficient of Variation (CV) values. The sensor measurement was completed by a 40 µl volume of electrolyte buffer (at the appropriate pH) was pipetted onto the applicator pad. The biosensor was left to equilibrate for 240 seconds prior to the addition of 20 µl of sample solution (diluted in phosphate buffer). Current output was recorded at 350 seconds and the net increase in current over the response recorded after 240 seconds was recorded. The working electrode was poised at 300 mV against Ag/AgCl. Values are shown ± SD (n=3).

L-malic acid biosensor			
	Mean L-malic acid (mM)	STDEV	CV* (%)
Tomatogratepres1	5.62	0.541	9.62
tomatogratepres2	4.66	0.312	6.69
tomatogratepres3	5.14	0.826	16.1
tomatofreezethow1	3.98	1.28	32.0
tomatofreezethow2	4.26	1.39	32.7
tomatofreezethow3	4.39	1.08	24.6
potatogratepres1	5.21	1.43	27.5
potatogratepres2	5.82	0.890	15.3
potatogratepres3	4.73	0.312	6.59
potatofreezethow1	5.07	1.43	28.3
potatofreezethow2	7.12	0.717	10.1
potatofreezethow3	6.03	0.890	14.8

*CV = (SD/ mean) x 100 %.

Table 5.3 Results determined obtained using L-malic acid test kit, giving the mean malic acid concentration in mM, standard deviation (SD), with the corresponding % value for the relative standard deviation (CV).

KIT RESULTS:			
Sample	L-malic acid (mM)	SD	CV
Tomato grate-press1	4.73	1.45	30.6
Tomato grate-press2	3.97	0.252	6.34
Tomato grate-press3	5	0.361	7.21
Tomato freeze-thaw1	4.7	0.361	7.67
Tomato freeze-thaw2	4.23	0.451	10.7
Tomato freeze-thow3	4.9	0.173	3.53
Potato grate-press1	6.7	0.173	2.59
Potato grate-press2	6.17	0.231	3.75
Potato grate-press3	5.47	0.451	8.25
Potato freeze-thaw1	4.1	6E-08	0
Potato freeze-thaw2	7.7	0.36	4.68
Potato freeze-thaw3	5.97	0.379	6.35

Results are shown in Table 5-4. Russell Burbank potatoes, mini plum tomatoes and Jonagold and Bramley apple varieties were found to have L-malic acid concentrations of 3-4 mM, 2.0-2.7 mM, 30-60 mM and 80-120 mM respectively.

The accuracy of the enzyme-electrode response was determined against the test kit method using the equation: Accuracy = 100% × ($[S_R-K_R] / K_R$); where S_R is sensor response and K_R is test kit response. In all cases, the sensor response was within 13.7% of the response of the standard method. Whilst improved accuracy would be desirable, the sensor method does provide a simple and field-based method for indicating the levels of L-malic acid in the tested horticultural samples, thus providing a more objective assessment of produce maturity and taste. The sensor method offers some advantages over the test kit method, particularly with regard to field-based measurements since the sensor merely requires the dilution and addition of prepared sample to the filter disc, whilst the test kit method requires a number of sample and reagent handling steps. Less training is required to operate the sensor method, which is more rapid (6 min. *vs.* 24 min.) with lower labour, disposables and reagent costs. However, the linear range of the sensor was 0.028 – 0.7 mM compared with 0.0038 mM – 2.5 mM for the test kit.

In addition to horticultural produce assessment, there may also be other potential applications for the L-malic acid biosensor. According to Palleschi *et al* (1998) the quality of red and white wines and their organoleptic characteristics are very much dependent upon the extent of internal malo-lactic fermentation. The net result of this type of fermentation is the formation of lactic and malic acids, which will influence the taste of the wine product.

Table 5.4 Comparison of sensor and standard photometric test kit results for measurement of L-malic acid in potato and apple samples. The sensor measurement was completed by a 40 µl volume of electrolyte buffer (at the appropriate pH) was pipetted onto the applicator pad. The biosensor was left to equilibrate for 240 seconds prior to the addition of 20 µl of sample solution (diluted in phosphate buffer). Current output was recorded at 350 seconds and the net increase in current over the response recorded after 240 seconds was recorded. The working electrode was poised at 300 mV against Ag/AgCl. Values are shown ± SD (n=3).

Sample	Dilution	Sensor [S_R] (mM)	Test kit [K_R] (mM)	Accuracy% [a]
potato (Russell Burbank)	×10	0.353 ±0.055	0.337 ± 0.019	4.8
potato (Russell Burbank)	×10	0.380 ±0.010	0.335 ± 0.096	13.4
potato (Russell Burbank)	×10	0.309 ±0.008	0.334± 0.09	-7.5
Apple (Jonagold)	×100	0.552 ±0.088	0.488 ± 0.086	13.1
Apple (Jonagold)	×100	0.479 ±0.059	0.542 ± 0.019	6.0
Apple (Jonagold)	×100	0.378 ±0.084	0.388 ± 0.081	-2.6
Apple (Bramley)	×200	0.391 ±0.008	0.419 ± 0.036	-6.7
Apple (Bramley)	×200	0.465 ±0.095	0.429 ± 0.012	8.4
Apple (Bramley)	×200	0.582 ±0.208	0.579 ± 0.124	0.5
Tomato (mini plum)	×10	0.234 ±0.124	0.269 ± 0.020	-13.0
Tomato (mini plum)	×10	0.201 ±0.035	0.233 ± 0.010	-13.7
Tomato (mini plum)	×10	0.270 ±0.047	0.258 ± 0.010	4.7

[A]Accuracy = $[(S_R - K_R)/K_R] \times 100$ %, where S_R is the sensor response and K_R is the test kit response.

Palleschi *et al.* (1998) further state that the biosensor approach matches the requirement of wine producers with regard to selective and rapid analysis of lactate and malic acid in wine.

The principal organic acid in natural apple juice is L-malic acid (0.15-0.91 % w/w) and no D-malic acid should be present. Since L-malic acid is expensive to produce, some commercial organisations have added synthetic D, L-malic acid to apple juice cartons and sold the product as pure apple juice. Governmental organisations require a simple method that can measure L-malic acid and therefore differentiate between synthetic and natural apple juice. In unadulterated apple juice, the L-malic acid:total malic acid ratio is 1.0, compared to 0.5-1.0 for adulterated juice. The current way of assessing apple juice adulteration is to measure total malic acid by HPLC, and L-malic acid using enzyme-based test kits Elkins and Heuser, (1994). The L-malic acid biosensor could have applications in place of the test kit where a limited number of simple, rapid measurements are preferred.

5.3.11 Cellulose acetate membrane:

In order to increase the compatibility between the dynamic range of the biosensor and the levels of L-malic acid commonly found in horticulture produce, methods to increase linear range were sought. Enzyme electrodes incorporating cellulose acetate membranes were prepared as described in the materials and methods section. The linear range was extended to 0.8 mM with 1 % cellulose acetate membrane (Figure 5.9). The equation of the line was $y = 0.2248x + 0.029$ while the R^2 value was 0.9757. This compared favourably with the normal L-malic acid biosensor (Linear

range 0.7 mM and equation of the line was y = 0.0924x + 0.0577). However the linear range was not increased significantly.

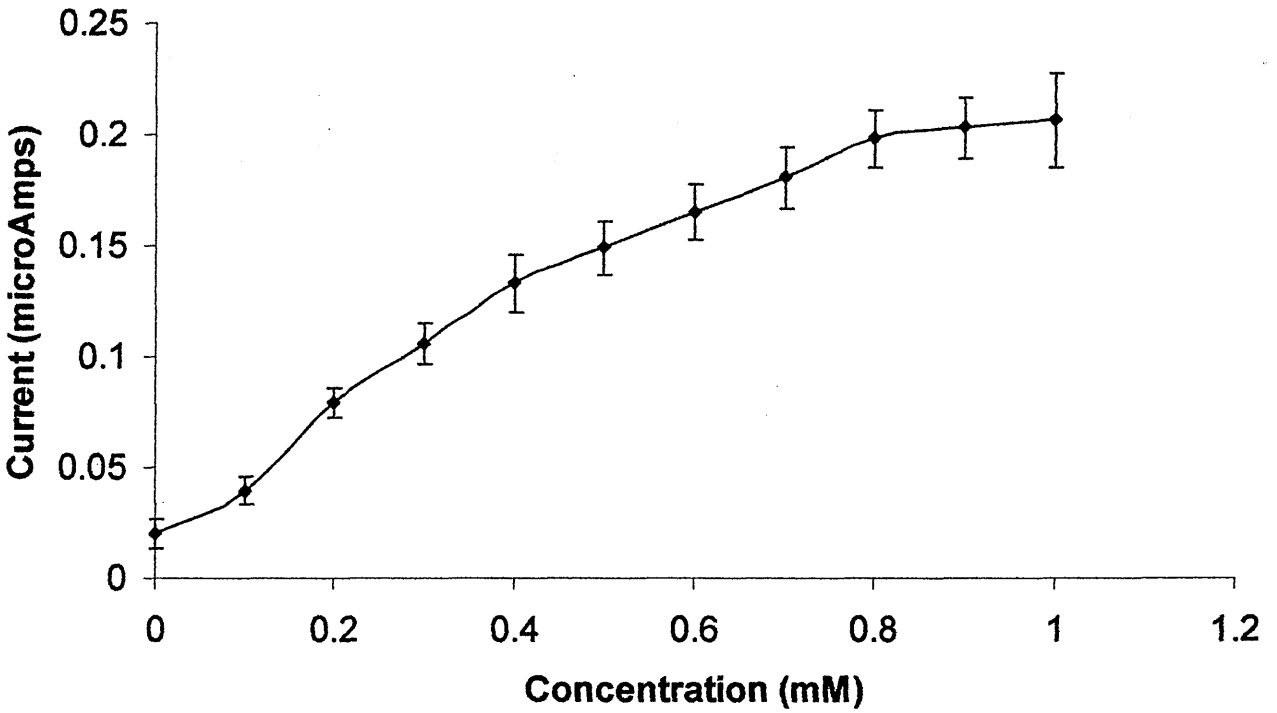


Figure 5.9 Calibration profile of L-malic acid biosensor coated with 1 % cellulose acetate. A 1% cellulose acetate (w/v) in acetone solution was prepared and the enzyme electrode was dip coated twice (one minute apart), in the solution and left to dry for forty five minutes. The electrode was tested in 10 ml of buffer electrolyte solution in a stirred beaker. Each point represents a means of three separate 1 % cellulose acetate membrane electrodes, at the same concentration. The electrode was poised at 300 mV against Ag/AgCl.

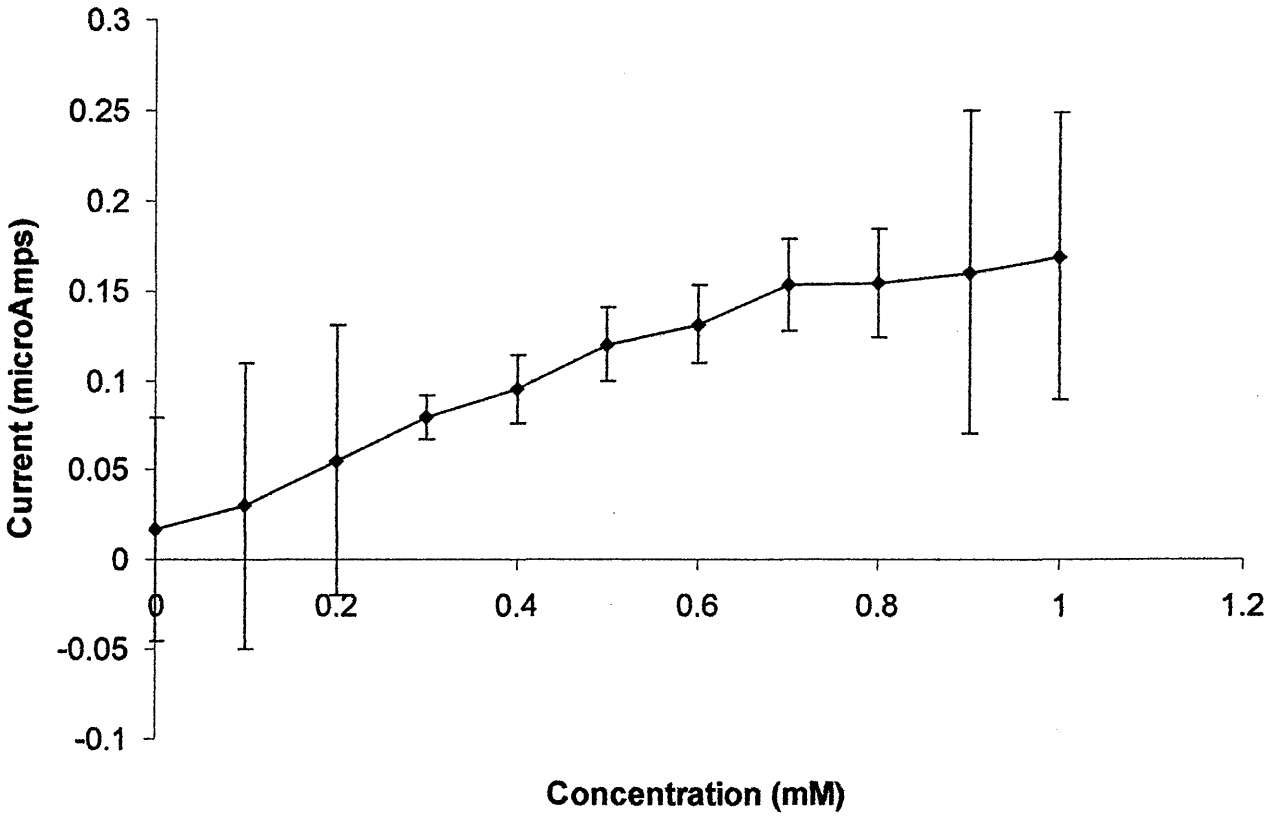


Figure 5.10 Calibration profile of L-malic acid biosensor coated with 2 % cellulose acetate. A 2% cellulose acetate (w/v) in acetone solution was prepared and the enzyme electrode was dip coated twice (one minute apart), in the solution and left to dry for forty five minutes. The electrode was tested in 10 ml of buffer electrolyte solution in a stirred beaker. Each point represents a means of three separate 2 % cellulose acetate membrane electrodes, at the same concentration. The electrode was poised at 300 mV against Ag/AgCl.

Therefore an approach using 2 % cellulose acetate membrane was undertaken. In this case the maximum range achievable remained below 0.8 mM. However the excessive noise was reported at low and high concentrations that made results highly inaccurate (Figure 5.10).

5.3.12 Malate dehydrogenase route

5.3.12.1 Selection of optimum measurement conditions

$$\text{L-malic acid} + NAD^{+} \overset{\text{MDH}}{\Leftrightarrow} \text{oxaloacetic acid} + NADH + H^{+}$$

The optimum pH values for free malate dehydrogenase (MDH, EC 1.1.1.37) and free glutamate oxaloacetate transaminase (EC 2.6.1.1, GOT) are pH 9.2 to 9.5 (for malate) and pH 6.5 to 8.5 (for glutamate), respectively. A high pH has an undesirable effect on the stability of these enzymes (Gilis *et al* 1996). Therefore pH 9 glycine buffer was used for all experiments. GOT catalyses the conversion of oxaloacetate and glutamate to 2-oxoglutamate and aspartate. The concentration of glutamate affects the rapid removal of oxaloacetate produced in the reaction catalysed by malate dehydrogenase. Matsumoto and colleagues (1996) were able show that the maximum response of the biosensor was achieved with 5 mM of NAD and 5 mM of glutamate.

5.3.12.2 Determination of sensor analytical performance

Initially the experiment was run in beaker tests with the enzymes being immobilised on the working electrode surface in HEC. However, a very poor correlation between sensor response, NADH concentration was observed across the desired analytical range ($y = 0.0407x + 0.169$ $R^2 = 0.361$).

An alternative methodology was developed whereby the sample solution was applied directly onto the screen-printed electrode pad, to minimise enzyme desorption issues. Again a very poor correlation between sensor response and NADH concentration was obtained ($y = -0.0877x + 4.3353$, $R^2 = 0.1087$).

The results indicated that the malic enzyme biosensor was far superior in detecting L-malic acid, as compared to the MDH biosensor approach. Possible reasons for this may include that the malic enzyme's equilibrium is towards the removal of L-malic acid. The equilibrium for MDH is towards the formation of L-malic acid; therefore other methods, such as immobilising GOT (section 5.1) are required to help shift the equilibrium. Consequently this makes the enzymatic process more complex. Further work may be required in future to optimise each of the parameters in order to improve sensor response.

Results reported in literature (*Matsumoto et al.*, 1996)) show that the linear range with the MDH route is between 0.01 mM to 0.05 mM, and below pH 9 the activity of the enzyme was significantly reduced. In this work one of the objectives is to measures several analytes simultaneously. Malic enzyme biosensor offered a higher linear range and a better pH range.

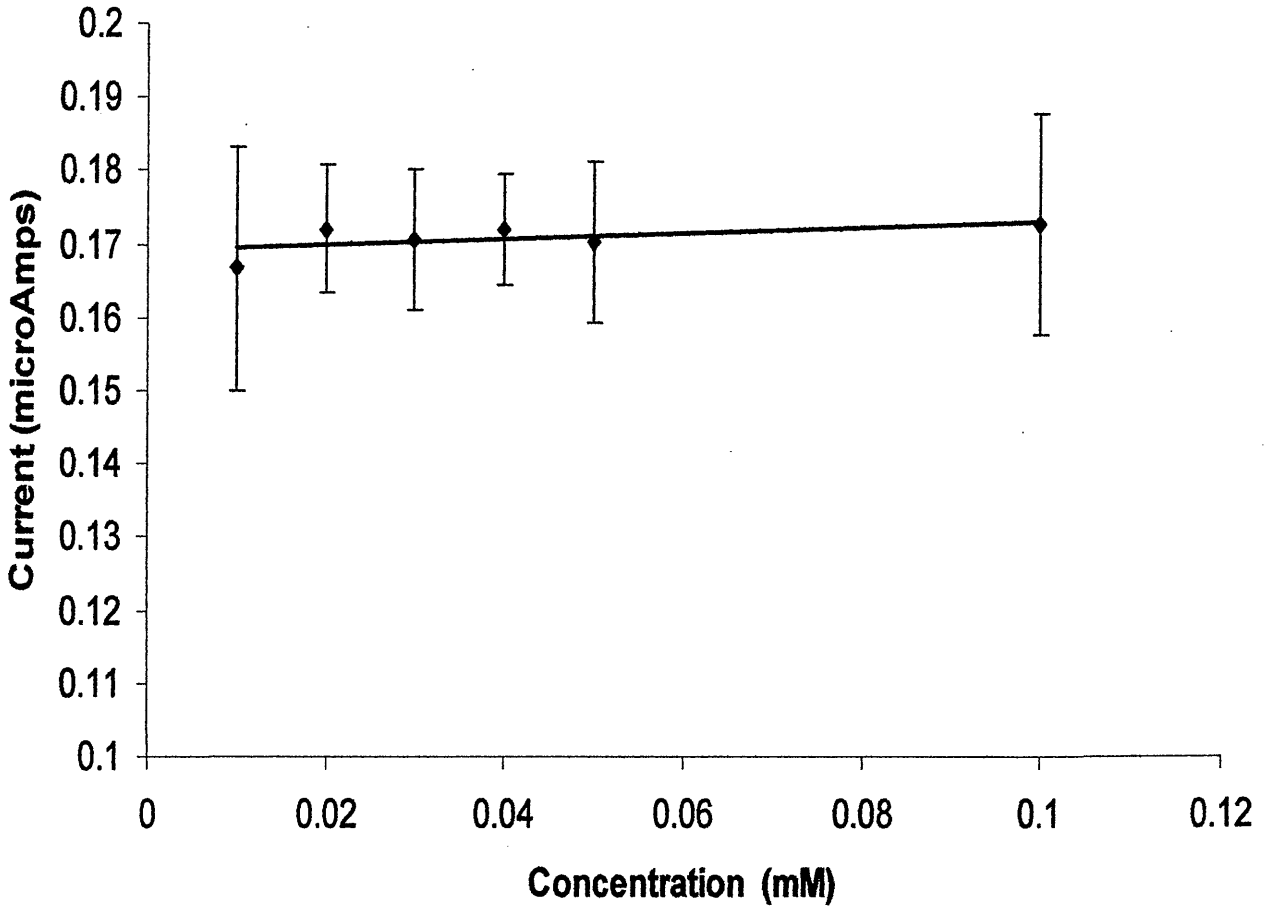


Figure 5.11 Calibration profile for determination of L-malic acid using immobilised malate dehydrogenase (MDH) and glutamate oxaloacetate transaminase GOT). The electrode was dipped in a stirred beaker containing 10 ml of glycine buffer. The electrode was poised at 300 mV against Ag/AgCl.

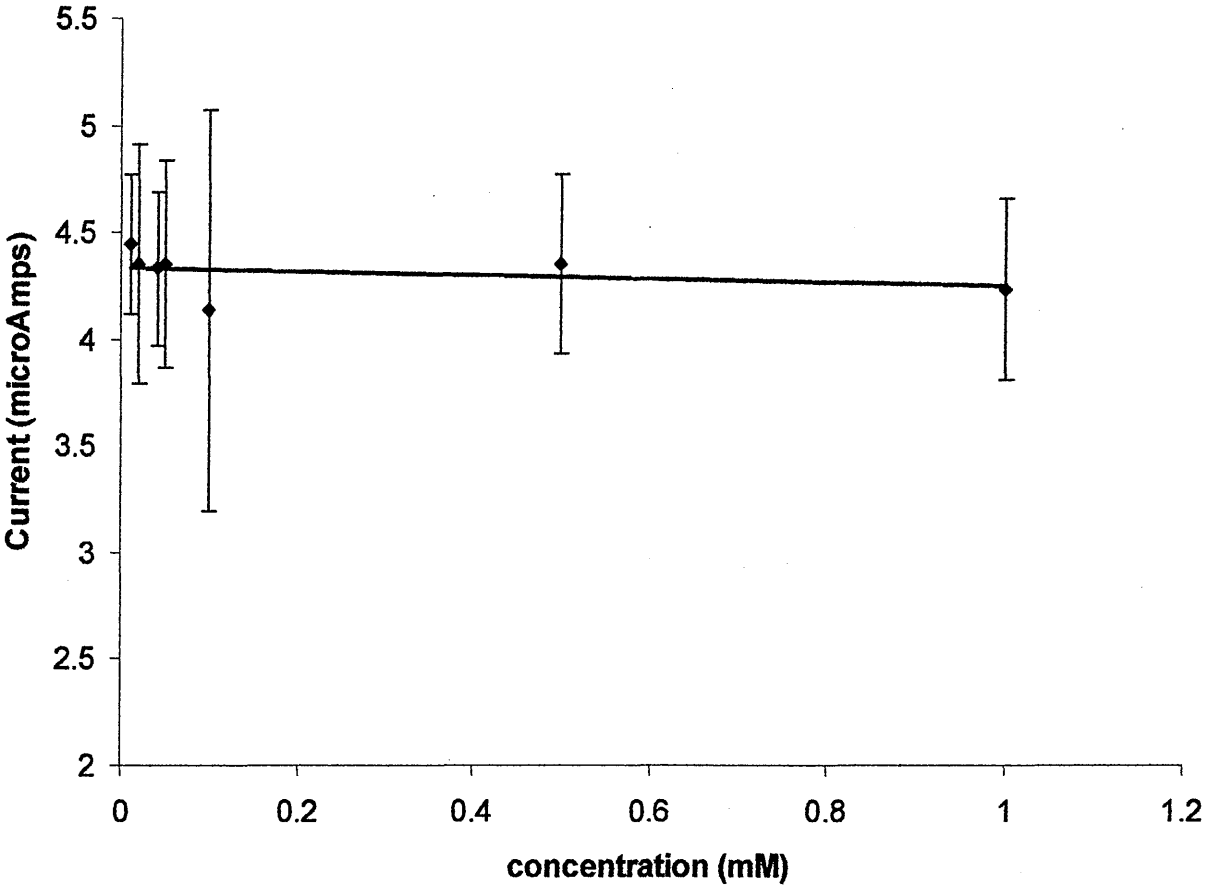


Figure 5.12 Determination of L-malic acid by droplet addition by use of malate dehydrogenase. MDH and GOT were co-immobilised on to the working electrode surface. 20 µl volume of L-glutamic acid (5mM), and 20 µl NAD^+ (5mM) was then applied to the membrane-electrode-assembly. The amperometric response was measured at a potential of 300 mV against Ag/AgCl. A 20 µl volume of L-malic acid was added 200 seconds after initial sample addition and a reading taken after a further 200 seconds.

5.4 Conclusions

Malate dehydrogenase (MDH) biosensors were subject to two measurement regimes, (i) sample addition to stired beakers; (ii) addition of a small volume of sample to an applicator pad overlaying the three electrode assembly. Both approaches proved inadequate in obtaining satisfactory analytical data with standard solutions.

Screen-printed electrodes based on malic enzyme with amperometric measurement of NADPH oxidation have proven suitable for the simple, low-cost and rapid analysis of L-malic acid in apple, potato and tomato samples. Addition of mediator is not required since the rhodinised carbon working electrode favoured the oxidation of NADPH at a lower operating potential (+300 mV vs. Ag/AgCl) compared with a number of possible organic acid interferents, excepting L-ascorbic acid. Residual interference effects can be accounted for through use of a compensator electrode. The sensor performance was tested against a photometric kit using real samples, yielding accuracy values within 13.7% of the standard method. Since the measurement process is simple, it is amenable to field-based usage with a minimal training requirement. Low sensor manufacturing costs result in single-use disposable devices, thus negating problems of progressive electrode fouling.

6 DEVELOPMENT OF BIOSENSORS FOR THE MEASUREMENT OF D-GLUCOSE, L-AMINO ACIDS, L-GLUTAMIC ACID AND L-ASCORBIC ACID

6.1 Introduction

Current methods in food analysis include spectrophotometry (Elkins and Heuser, 1994), and liquid chromotography (Lee and Wrolstad, 1988). Chromatographic methods currently in use to determine sugars and amino acids are high-performance liquid chromatography, gas chromatography and capillary electrophoresis. Nevertheless, chromatography of sugars often suffers from low sensitivity and time–consuming processes (18 minutes analysis time). The necessity of other detection methods, such as mass spectrometry is necessary especially in cases where natural matrices are present (Molnar-Perl, 2000). On the other hand, the impact of capillary electrophoresis on food science has had limited acceptability due to detection difficulties (Soga *et al.*, 1998). High-performance liquid chromatography is a successful approach for amino acid detection. One advantage of chromatography is that it is able to separate and quantify individual amino acids. This approach is often hindered by the absence of a strong chromophore, and formation of derivatives is often required to enhance the absorbivity (Casella *et al.*, 2000). Photometric detection of amino acids upon chemical derivatisation with o-phthaladehyde (Abecassis *et al.*, 1985)

and ninhydrin (Hamilton, 1963) are very sensitive. All the above tests induce disadvantages, which includes the use of complex and expensive equipment and requirement of qualified personal.

Biosensors are useful measuring tools that can play a prominent part in food processing and quality control, because of their compactness in size, portability and specificity for a given analyte. Furthermore, electrochemical transduction has proved to be the most popular method, with amperometry being the most favoured configuration. Electrochemical biosensors can also offer other advantages over the photometric approach in that coloured or turbid samples do not interfere with the electrochemical response (Scouten *et al.*, 1995).

Analytes of interest for biosensor detection can include glucose which is naturally present in fresh produce and concentrations higher then 1.6 mg g^{-1} in the Russet Burbank variety of potatoes have been known to effected the frying colour of chips (Pritchard *et al.*, 1994). Total amino acids can be a partial indicator of food maturity, for example in apples the levels of amino acids decreases as the fruit matures (Ackermann *et al.*, 1992).

L-glutamic acid is also used in food additives in the form of monosodium glutamate. Measurement of individual analytes in food may not provide a clear view of the sensory characteristics of food. For this reason multi-analyte measurement is necessary.

Various types of amperometric biosensors for L-glutamic acid have been described and these include the immobilisation of glutamate oxidase (Ye *et al.*, 1995). This methology includes two possible disadvantages: It is currently an expensive enzyme for use in one-shot biosensors, and the enzyme has slight cross-reactivity with L-aspartate and glutamine (Zilkha *et al.*, 1995). Another method for measurement of L-glutamate is the use of L-glutamate dehydrogenase (GluDH), this enzyme has the advantages of being fast acting and also a high affinity for L-glutamate (Liu *et al.*, 1999).

This work involves the use of glutamate dehydrogenase (GD) and a cofactor NADP to measure L-glutamic acid.

$$\text{L-Glutamate} + \text{NADP+} + H_2O \xleftrightarrow{GD} \alpha\text{-ketoglutarate} + \text{NADPH} + NH4^+$$

Glucose has been measured by various enzymatic schemes including the use of glucose dehydrogenase (Turner *et al.*, 1987). However, glucose oxidase (GOD) is by far the most widely used enzyme in biosensors (Wilson and Turner, 1992). This is because this enzyme offers the advantages of high specificity, high turn over and has been found to be highly stable. The following reaction was used for subsequent methods:

$$\beta\text{-D- glucose} + O_2 \xrightarrow{GOD} \text{d-glucono-}\delta\text{-lactone} + H_2O_2$$

A common approach for biosensor measurement of amino acids has included the use of the enzyme amino acid oxidase. This enzyme has been known to be coupled to mediators where the reoxidation of the electron acceptor is measured amperometrically. As an alternative to mediation the use of metallised carbons to allow the electrochemical oxidation of peroxide detection at reduced operating potentials has been widespread in such systems (Sarkar *et al.*, 1999).

$$\text{Amino acid} \xrightarrow{\text{AAO}} \text{2-oxoacid} + NH_4^+ + H_2O_2.$$

In this chapter the development of screen-printed sensors for D-glucose, L-amino acids, L-ascorbic acid and L-glutamic acid is reported.

6.2 Materials and Methods

Reagents

All chemicals were supplied by Sigma™ (Poole, Dorset UK), unless otherwise stated. Water was purified by reverse osmosis (RO) using an Elgastat system (The Elgastat Group, High Wycombe, Bucks, England). Phosphate buffer (pH: 7.4, NaH_2PO_4-Na_2HPO_4) was prepared at a concentration 1M and 100 mM, containing 100 mM potassium chloride to stabilise the reference potential and act as an electrolyte.

6.2.1 Horticulture preparation

All real samples, unless stated, were provided by HRI (Horticulture Research International, Wellesbourne, Warwick, UK). These batches included tomato

samples of the Mini-plum variety, potato samples of the Russel Burbank variety and apple samples from the Jonagold variety and the Bramley variety. The potato variety Russet Burbank (RB) was selected as it is a popular variety of potatoes for making chips. Two batches of potato samples were tested. The two batches were named as RB1 and RB2, according to the month of delivery. The method of preparation of samples is described in chapter 4.

6.2.2 Sensor fabrication

6.2.3 Construction of the biosensors

The screen-printed electrodes were printed using the same method as described in chapter 5

6.2.3.1 Immobilisation of enzymes

All enzymes were adsorbed on the working electrode surface. Aliquots of 5 µl of L-amino acid oxidase (*Crotalus adamanteous* type IV, activity: 380U/mg or 530U/mg) and 1.842 µl of glucose oxidase solutions (*Aspergillus niger*, 8156 U/ml, supplied by Biozyme™, Blaenavon, U.K.), prepared in phosphate buffer (pH:7.4, 10 mM, KCl: 100 mM), were air-dried for 2-3 hours and stored at 4°C. Glutamate dehydrogenase (E.C. 1.4.1.3) was obtained from Sigma (Pool, Dorset, UK). The enzyme was in powder form that also contained buffer salts. Enzyme (11.37µg) was dissolved in 200 µl of reverse osmosis water. ADP is known to activate Glutamate dehydrogenase and therefore a 2 mM concentration of ADP (Sigma) was dissolved in the enzyme solution. Aliquots

(4 µl) of the resultant solution was pipetted onto the working electrode surface. The electrodes were air dried for 1 hour and were stored at 4°C until required for usage. A solution containing 0.1 M potassium chloride and 10 mM NADP (Sigma, Pool, Dorset, UK), was prepared in 0.1 M phosphate buffer at pH 7.4. In the case of L-ascorbic acid biosensor aliquots of 10 µl of Ascorbate oxidase (Sigma, Pool, Dorset, UK), (30 units per ml), was pipeted onto the working electrode. The electrodes were then air dried for half an hour, and stored at 4 °C until required for usage.

6.2.3.2 Test procedure

The test procedure was controlled using an Autolab Electrochemical Analyser with GPES3 software (Ecochemie, Utrecht, The Netherlands). A 1.1 cm diameter Whatman 114 filter disc (Whatman Ltd., Maidstone, UK) was placed over the 3-electrode assembly which, when wetted with sample, completed the electrochemical circuit. It should be noted that real samples were prepared in 0.1 M phosphate buffer containing 0.1 M KCl in order to ensure consistent solution pH and supporting electrolyte concentration.

6.2.3.3 Amino Acid procedure

A 40 µl sample was deposited onto the filter paper and the working electrode poised at a potential of +350 mV versus the Ag/AgCl reference. The system was allowed to equilibrate in the presence of sample, according to the method of Kröger *et al.*, (1998), and the current value noted at 4 minutes. Tests were performed at ambient temperature on screen-printed electrodes unless otherwise stated. Amino acid tests solutions were prepared in buffer-electrolyte.

6.2.3.4 Glucose Procedure

A 30 µl volume of buffer was deposited onto the filter paper and the working electrode poised at a potential of 350 mV versus the Ag/AgCl reference. After 110 seconds equilibration time, 30 ml of a sample was added. Readings were taken 240 seconds after the addition of the sample.

6.2.3.5 L-glutamic acid procedure

Sample solution (40 µl) was deposited on the filter paper and the enzymatically generated NADPH was measured at 240 seconds. The working electrode potential was maintained at a potential of +300 mV against the Ag/AgCl reference electrode.

6.2.3.6 L-ascorbic acid procedure

Cotton mesh was soaked in 10 ml of electrolyte buffer (phosphate buffer pH 7, containing 100 mM KCl). This was inserted onto the screen printed electrode. 500 µl of known concentrations of L-ascorbate acid was added to the applicator pad. The working electrode potential was maintained at a potential of +300mV against the Ag/AgCl reference electrode. At 150 seconds the current was measured.

6.2.3.7 Ninhydrin test method

Test reagent: 150 ml glycerol, 0.625 g ninhydrin, 18.387 g citric acid, 133.3 µl of 150 mM $MnSO_4$, made up to 250ml in water. The test reagent (2 ml) was added to 0.1 ml of sample diluted 10-fold in water. The mixture was heated at 100 °C for 12 min., diluted 2-fold in water and the optical density recorded at 570 nm. The method works for all amino acids except proline.

6.2.3.8 Photometric analysis

Methods for the spectrophotometric detection of D-glucose and l-glutamate are described in chapter 4. L-ascorbic acid were measured spectrometrically using a commercially available food test kit supplied by Diezyme, Oxford UK (cat No. 0409677). The method is shown below.

The test kit contained three consituents. Bottle one (43 ml) consisted of sodium phosphate/citrate buffer and also MTT {3 – (4,5 – dimethylthiazolyl -2) – 2,5 – diphenyltetrazolium bromide]. Tube two contained 20 ascorbate oxidase spatulas, 17 U ascorbate acid oxidase (AAO) each. Bottle three contained 4 ml PMS (5 – methylphenazinium methosulphate).

For each sample two cuvettes were used, one containing the sample and the other was the blank cuvette. In both cuvettes 1 ml of solution from bottle one was added. 0.1 ml of sample solution was also added with 1.5 ml of reverse osmosis (RO) water. However a spatula from tube 2 was only inserted into the blank quvette. The samples were mixed and incubated for six minutes at 37 °C. Absorbance at 578 nm was read or both quvettes. The reaction was then started by pipetting a volume of 0.1 ml solution to each of the quvettes. The quvettes were then remixed, and left to stand at 37 °C for a further 15 minutes. The absorbances of sample blank and also the sample quvette were read immediately one after the other at 578 nm.

6.3 Results and Discussion

6.3.1 Calibration of sensor response

Calibration curves were constructed with individual analytes in order to determine the linearity of the substrates. The results were compared to test kit analysis.

6.3.1.1 L-glutamic acid

Glutamic acid was able to give a linear response up to a concentration of 0.5 mM (Figure 6.1). The Limit of Detection (LOD) value for the glutamate sensor was considered to be twice the standard deviation of the biosensor with no substrate addition. This value was added to the sensor response and the values were converted to concentration units, the limit of detection was 0.198 mM and therefore was only active over a narrow range. The sensor was able to give a correlation (r) of 0.985 and the equation of the line was $y = 0.1073x$. Coefficient of variation (CV) ranged from 11% to 25%. For the fruit test kits the equation of the line was $y = 0.5843x + 0.0155$. CV ranged between 1% and 7%. The limit of detection was observed to be 0.007 mM. A correlation between test kit and biosensor was determined (Figure 6.2). This has shown a degree of correlation between the biosensor and test kit, ($r = 0.982$ and the equation of the line was $y = 0.1904x - 0.0044$), nevertheless, from the intercept values it was seen that there was a disparity between both methods.

6.3.1.2 D-glucose

Initial findings have been able to determine that the linear range for glucose was able to extend to 10 mM with a correlation factor $R = 0.997$ (Figure 6.3). The equation of the line was $y = 0.404x + 0.175$. CV values for the glucose biosensor ranged from 2

% to 14 %. Limit of detection was found to be 0.17 mM. However, the photometric response was able to give CV values of 0 % and a correlation of 1. The equation of the line was 0.2153x. The limit of detection of this method was 1.1154 μM. It can be seen from the results that the glucose test kit is superior to the biosensor in terms of accuracy however in relation to forming a sensor that would achieve rapid routine testing the glucose biosensor can prove adequate. A correlation factor of 0.998 was measured by comparing biosensor and photometric responses (Figure 6.4). The equation of the line was y = 2.1116x +0.0608

6.3.1.3 L-Amino acids

L-Amino acid oxidase enzyme is able to detect a range of Amino Acids and has specific specificities for certain Amino Acids. Therefore, Standard curves were plotted for L-Leucine, and L-phenylalanine, which are known to have a high precision for the enzyme (Bennetto *et al.*, 1987). Both Amino Acids were shown to exhibit linearity up to 1mM, and the correlation between L-leucine (Figure 6.6) and L-phenylalanine (Figure 6.5) was 0.989 and 0.998 respectively. The equation of the line for L-leucine was y = 2.001x - 0.100. CV values for the amino acid ranged from 3% to 16%. Phenylalanine was able to exhibit a gradient of 1.501 and an intercept value of 0.002, showing that phenylalanine and leucine gave low background interference. Both amino acids gave steep slopes when compared to other analytes. However L-leucine has a greater amperometric response, and this may be explained by L-leucine having a higher sensitivity towards L-amino acid oxidase as compared to L-phenylalanine.

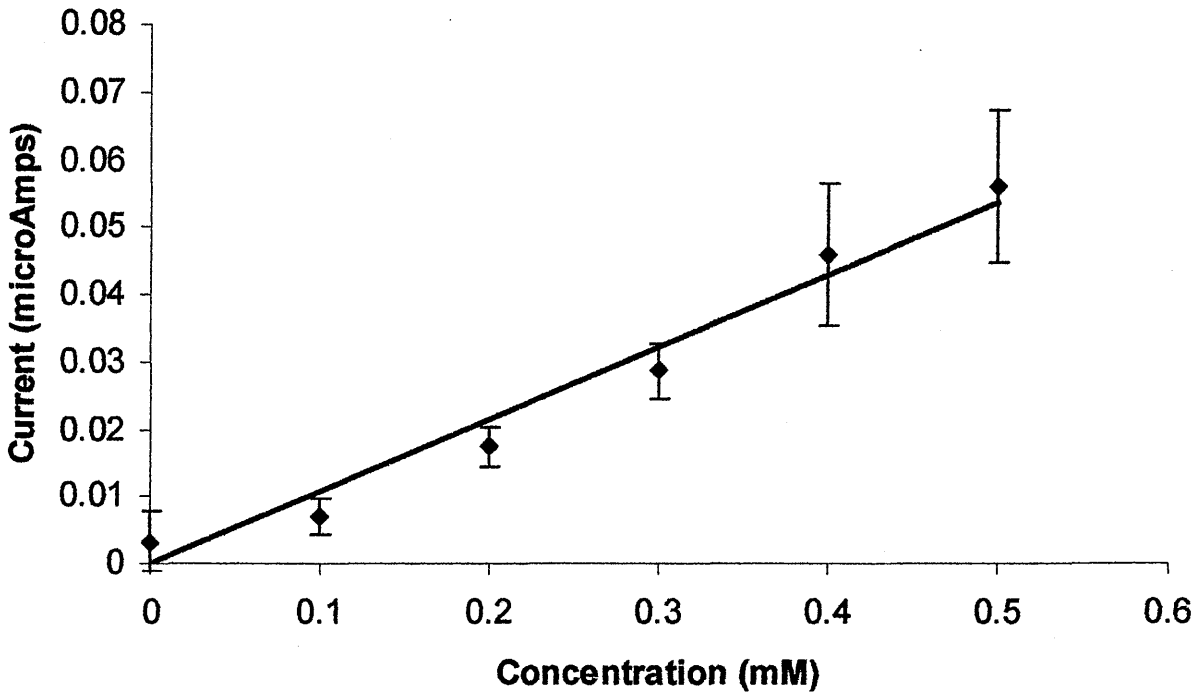


Figure 6.1 Current (y axis) verses concentration (x axis) profile of L-glutamic acid biosensor. For the biosensor analysis, L-glutamic acid (40 µl) in 0.1 M buffer electrolyte was deposited on the applicator pad and the enzymatically generated NADPH was measured at 240 seconds. The working electrode potential was maintained at a potential of + 300 mV against Ag/AgCl reference electrode. The samples were tested in triplicate.

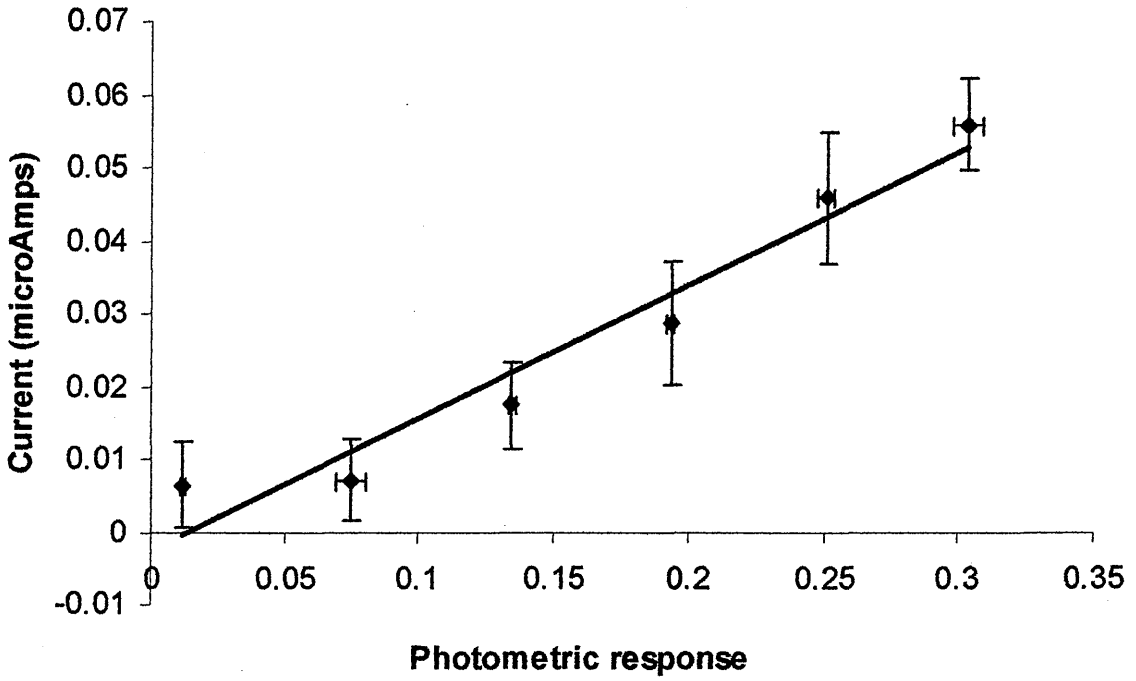


Figure 6.2 Glutamate biosensor response (y axis) compared to photometric response (x axis). For the biosensor analysis, L-glutamic acid (40 µl) in 0.1 M buffer electrolyte was deposited on the applicator pad and the enzymatically generated NADPH was measured at 240 seconds. The working electrode potential was maintained at a potential of + 300 mV against an Ag/AgCl reference electrode. The samples were tested in triplicate. Both tests measured concentrations up to 0.5 mM. The optical density for the photometric response was 340 nm.

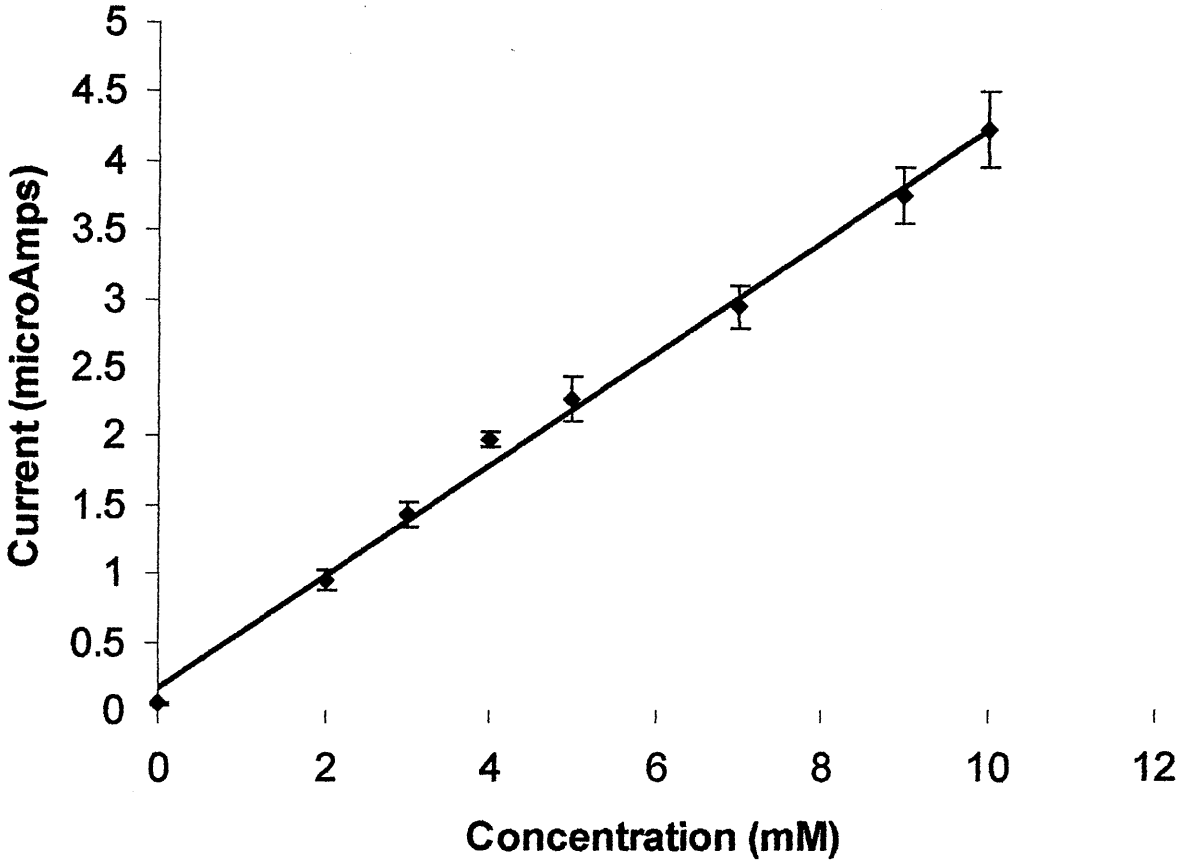


Figure 6.3 Current (y axis) verses concentration (x axis) profile of D-glucose biosensor. A 30 µl volume of buffer (phosphate buffer (100 mM) pH 7.4 containing 100 mM KCl) was deposited onto the applicator pad and the working electrode poised at a potential of 350 mV versus the Ag/AgCl reference. After 110 seconds equilibration time, 30 ml of a sample (known concentration of D-glucose) was added. Readings were taken 240 seconds after the addition of the sample. The samples were tested in triplicate.

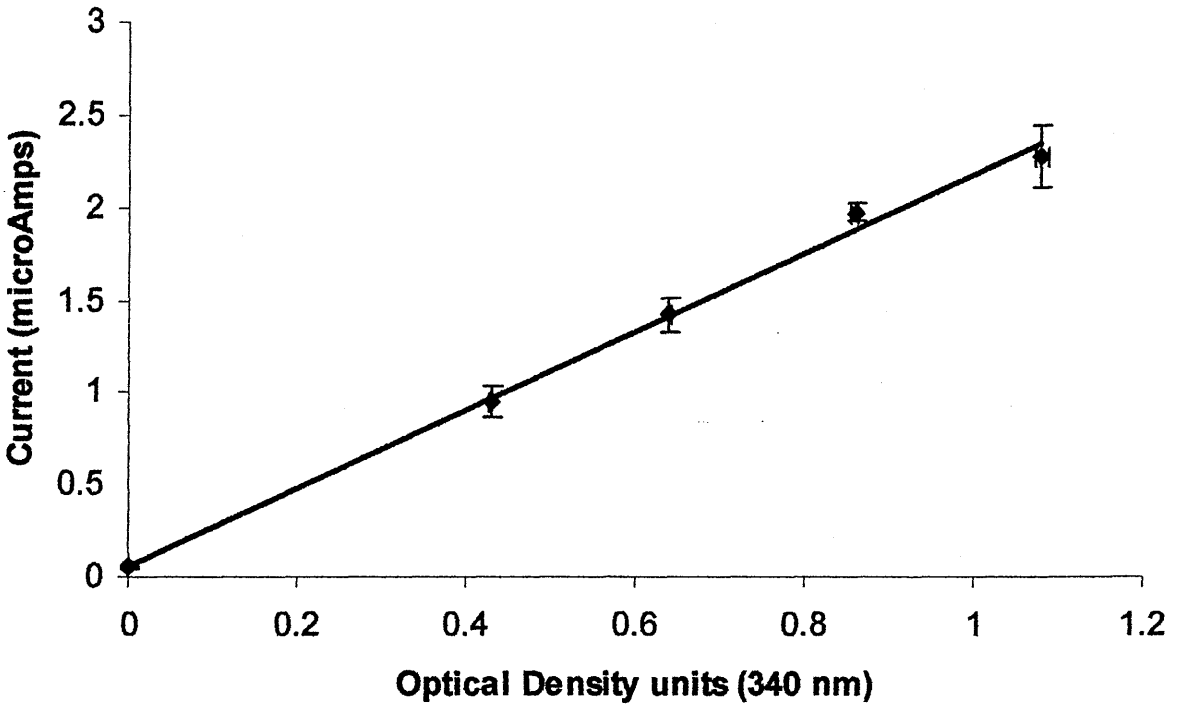


Figure 6.4 Glucose biosensor response (y axis) compared to photometric response (x axis). A 30 µl volume of buffer was deposited onto the filter paper and the working electrode poised at a potential of 350 mV versus the Ag/AgCl reference. After 110 seconds equilibration time, 30 ml of a sample was added. Readings were taken 240 seconds after the addition of the sample. The samples were tested in triplicate. Both tests measured concentrations up to 5 mM. The optical density for the photometric response was 340 nm.

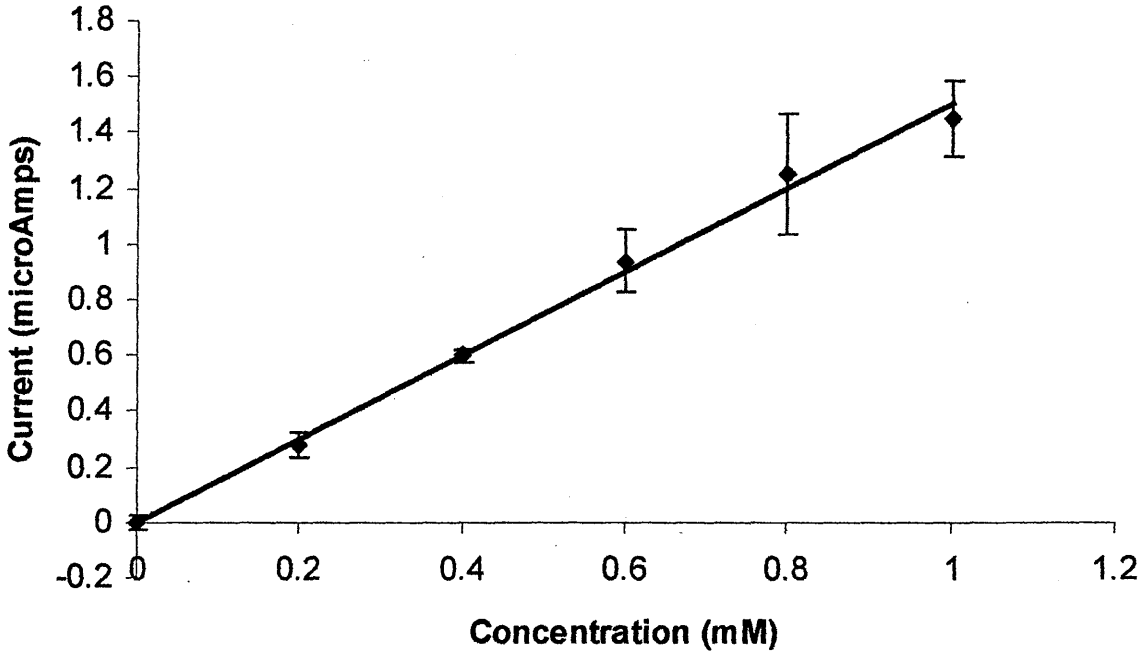


Figure 6.5 Current (y axis) verses concentration (x axis) profile of L-phenylalanine biosensor. A 40 µl known amount of L-phenylalanine in 100 mM phosphate buffer (pH 7.4, 100 mM KCl) was deposited onto the applicator pad and the working electrode poised at a potential of +350 mV versus the Ag/AgCl reference. The system was allowed to equilibrate in the presence of sample. The samples were tested in triplicate.

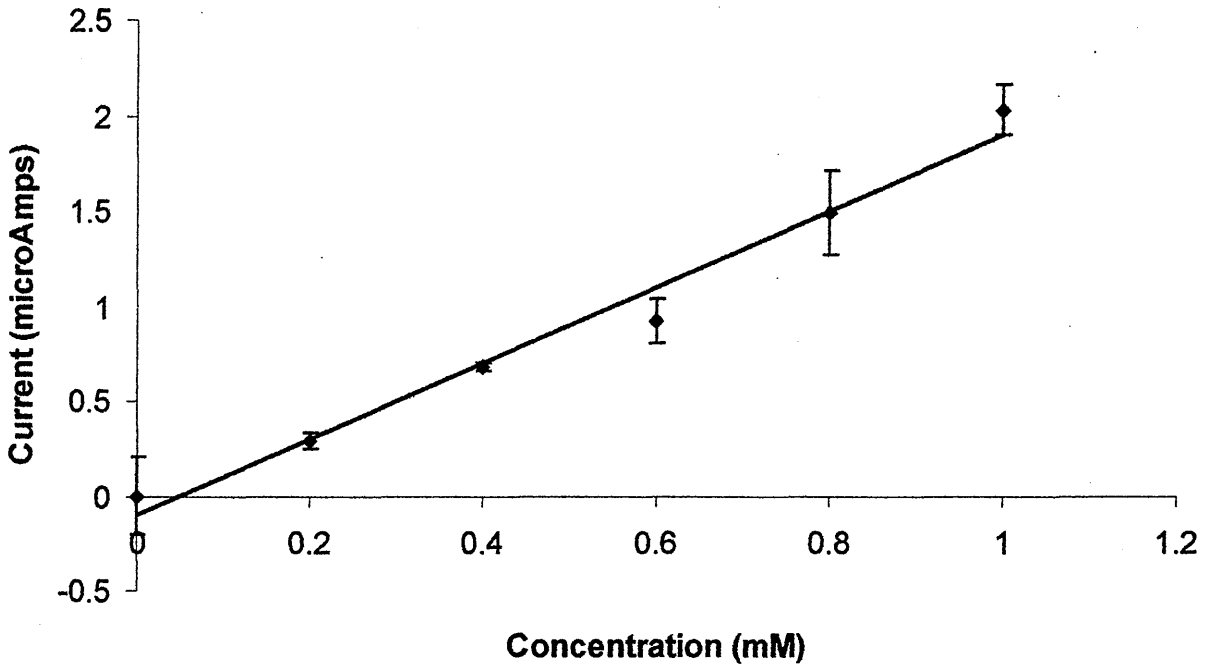


Figure 6.6 Current (y axis) verses concentration (x axis) profile of L-leucine biosensor. A 40 µl known amount of L-leucine in 100 mM phosphate buffer (pH 7.4, 100 mM KCl) was deposited onto the applicator pad and the working electrode poised at a potential of +350 mV versus the Ag/AgCl reference. The system was allowed to equilibrate in the presence of sample. The samples were tested in triplicate.

CV values for phenyalanine ranged from 2% to 17%. As far as the author knows there are no commercially available test kits that are able to quantify amino acids. As a result the ninhydrin test provided a standard photometric method for the quantification of amino acids. Ninhydrin was able to give a linear range up to 1 mM and the equation of the line was $y = 0.2401x + 0.0254$ for L-Leucine. Conversely a correlation of 0.981 was accomplished. Figure 6.7 is able to show a comparison between the ninhydrin method and biosensor measurements for L-leucine. A high degree of correlation was evident between both methods ($R= 0.994$).

6.3.1.4 L-Ascorbic acid

In the case of the L-ascorbic acid biosensor, none of the products of the L-ascorbate oxidase reaction were electroactive. Therefore measurement of L-ascorbic acid was performed, by determining the reduction of the electroactive substrate (L-ascorbic acid). This was achieved by a differential measuring technique where the sample is measured both in the presence and absence of enzyme. As far as the author knows Kriz *et al.*, (2001) are the only group that have employed a similar strategy in measuring Ascorbic acid, for biosensor analysis.

$$\text{L-ascorbic acid} + 0.5\ O_2 \xrightarrow{\text{L-ascorbate oxidase}} \text{dehydroascorbic acid} + H_2O$$

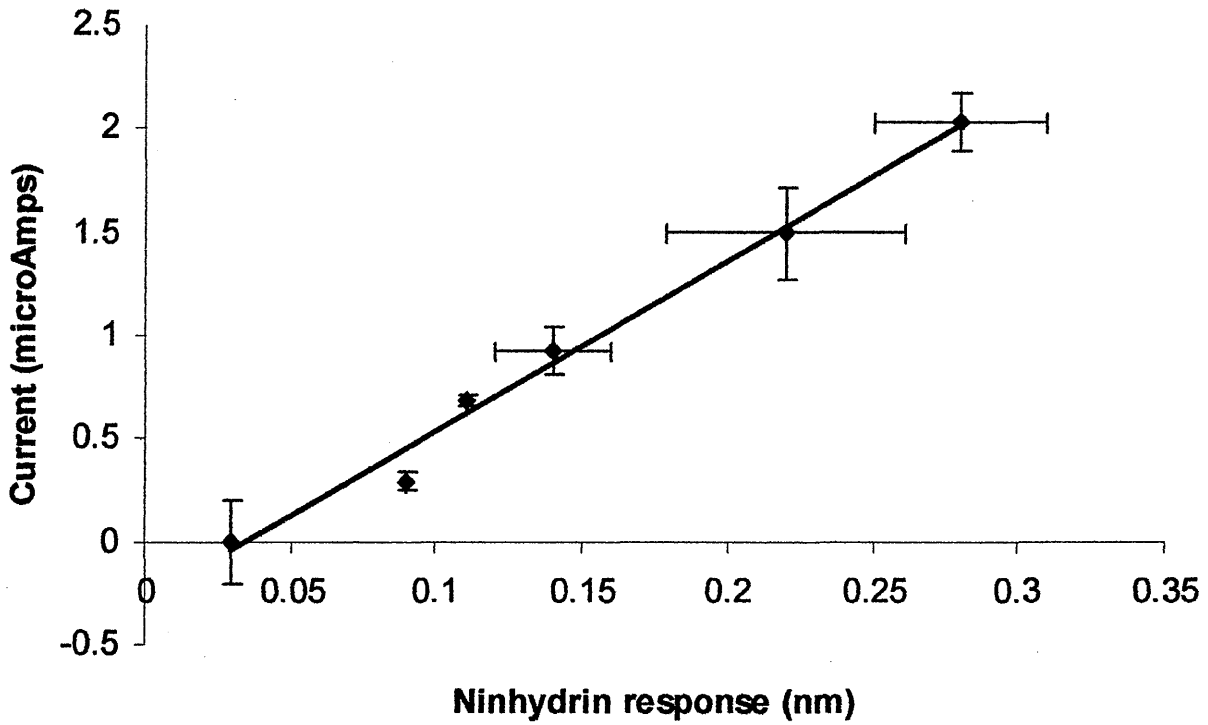


Figure 6.7 Amino acid biosensor response (y axis) compared to photometric response (x axis). A 40 µl sample was deposited onto the applicator pad and the working electrode poised at a potential of +400 mV versus the Ag/AgCl reference. The system was allowed to equilibrate in the presence of sample. The samples were tested in triplicate. Both tests measured concentrations up to 1 mM. The optical density for the photometric response was 570 nm.

The method described here is also analogous to the L-ascorbic acid test kit method, where, PMS reacts with L-ascorbic acid and also other reducing substances (blank quvette). The reduced MTT – formazan is able to give a signal at 578 nm. In the sample cuvette, L-ascorbic acid is converted to dehydroascorbate and therefore, in this case, the production of reduced MTT – formazon accounts for the reducing substances present in the sample. Subtraction of blank value from the sample value accounts for the amount of L-ascorbic acid present.

$$\text{L-ascorbate} + \text{MTT} \xrightarrow{\text{PMS}} \text{dehydroascorbate} + \text{MTT-formazan} + H^+$$

The work in this section is concerned with optimising parameters for the measurement of L-ascorbic acid.

6.3.1.4.1 Peak potential

Initially the peak working potential of the biosensor for the detection of L-ascorbic acid was measured. Using step amperometry positive potentials ranging from 0.3 mV to 0.6 mV against Ag/AgCl, were used to determine peak current values. The test was repeated with buffer electrolyte solution. The ratio of L-ascorbic acid and buffer electrolyte is shown in Figure 6.8. The highest ratio was achieved at 0.3 mV, while ratio was minimal at 0.6 mV. For this experiment a potential of 0.3 mV was chosen.

6.3.1.4.2 Peak enzyme concentration

The enzyme in this case is not reused therefore the amount needed for the measurement was optimised. The influence of the ascorbate oxidase on the

differential response was measured from 0-30 U per ml of ascorbic oxidase. There was a decrease in current response with increasing quantity of enzyme. Above 20 units the response of the biosensor was analogous to the response of buffer electrolyte with no enzyme. An excess of enzyme was chosen at a working concentration of 30 units, in order that the enzyme concentration would not be the limiting factor for analyte conversion. The results are shown in Figure 6.9.

6.3.1.4.3 Determination of sensor analytical performance

The linear range of the L-ascorbic acid biosensor containing L-ascorbate oxidase was determined. A parabolic relationship was obtained where the equation of the line was y = 0.7179x4 - 0.8688x3 + 0.3493x2 - 0.0585x + 0.2954. The correlation coefficient (r) was 0.99373. The principle of the biosensor was that L-ascorbate oxidase would remove free ascorbic acid in the sample and thereby the current would be comparable to the buffer electrolyte containing no-enzyme. It was further presumed that the detectable biosensor response would fall between the maximum and minimum standard deviation of the buffer electrolyte. With these criteria, the biosensor was able to measure L-ascorbic acid up to 0.6 mM. An L-ascorbic acid standard curve, containing no enzyme, was also constructed where the limit of detection was determined as 0.03 mM. The equation of the line was y = 5.0644x + 0.1305 where the coefficient of correlation (r) was 0.9943. A standard curve for L-ascorbic acid photometric test kit was also constructed, the limit of detection was found to be 0.005 mM. The equation of the line was y = 0.5351x.

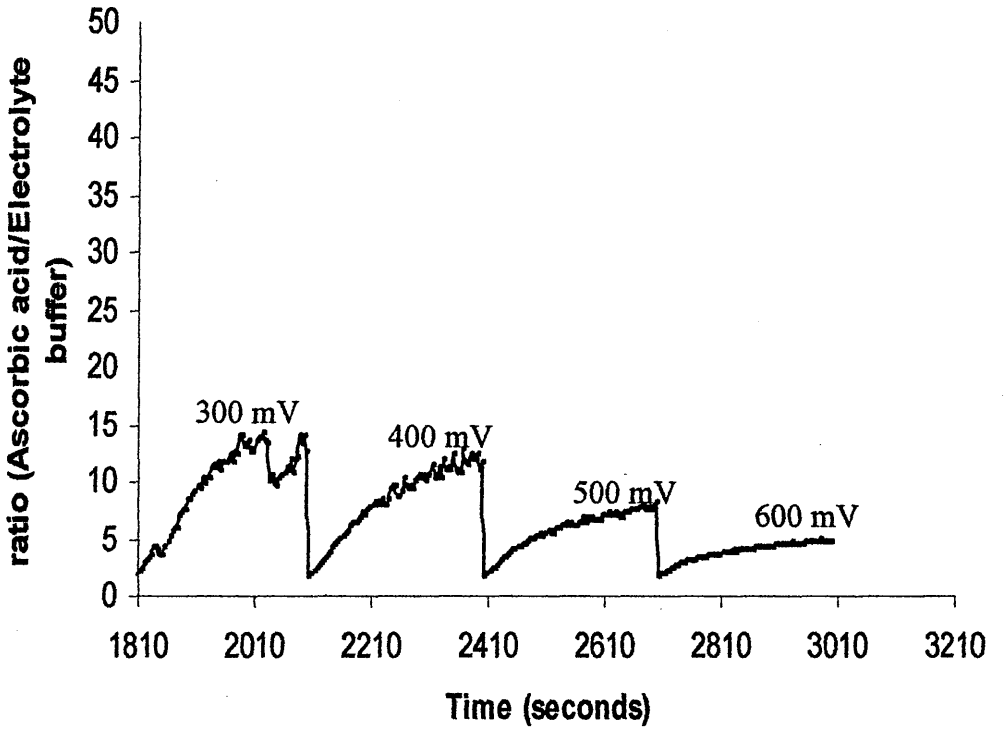


Figure 6.8 step up amperometric measurement peak potential of L-ascorbic acid. The potential was measured by dipping a screen printed electrode (enzyme free) in 10 ml volume of buffer electrolyte (100 mM phosphate buffer pH 7, incorporating 100 mM KCl) containing 0.1 mM of L-ascorbic acid. The potential range was between 0.3 mV to 0.6 mV. The sample was run in triplicate.

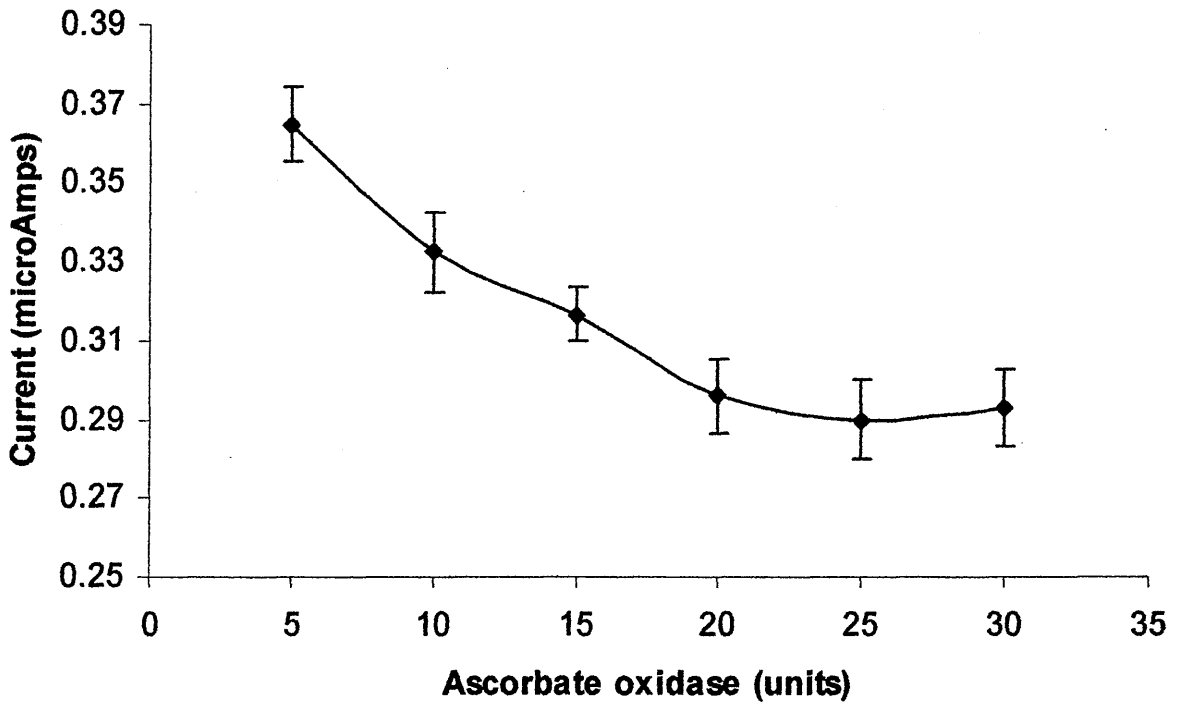


Figure 6.9 Influence of different ascorbate oxidase concentrations on the response of the biosensor. 500 µl of L-ascorbate acid (0.9 mM), in phosphate buffer (100 mM), containing 100 mM KCl, was pipetted to the applicator pad. The working electrode potential was maintained at a potential of +300mV against Ag/AgCl reference electrode. At 150 seconds the current was measured. The experiment was conducted in triplicate.

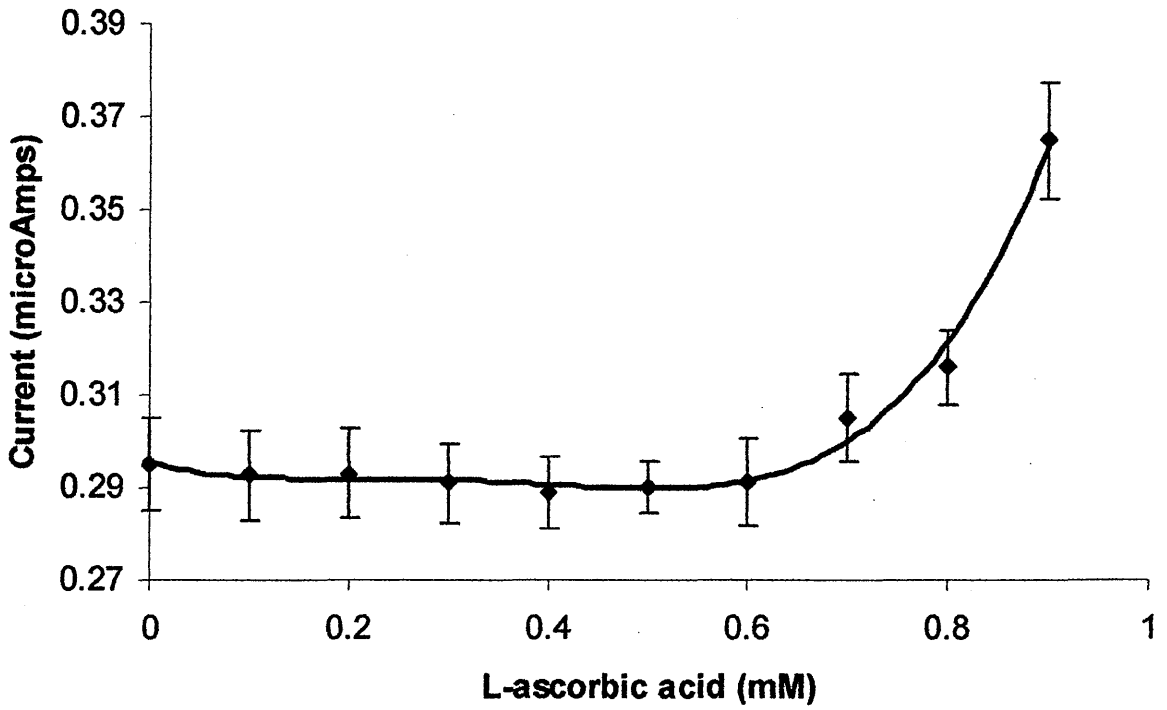


Figure 6.10 Detectable range of ascorbate oxidase biosensor. 500 µl of known concentrations of L-ascorbate acid, in phosphate buffer (100 mM), containing 100 mM KCl, was pipetted to the applicator pad. The working electrode potential was maintained at +300mV against Ag/AgCl reference electrode. At 150 seconds the current was measured. The experiment was performed in triplicates. 30 units of ascorbate oxidase were used. pH 7 phosphate buffer was employed in the experiment.

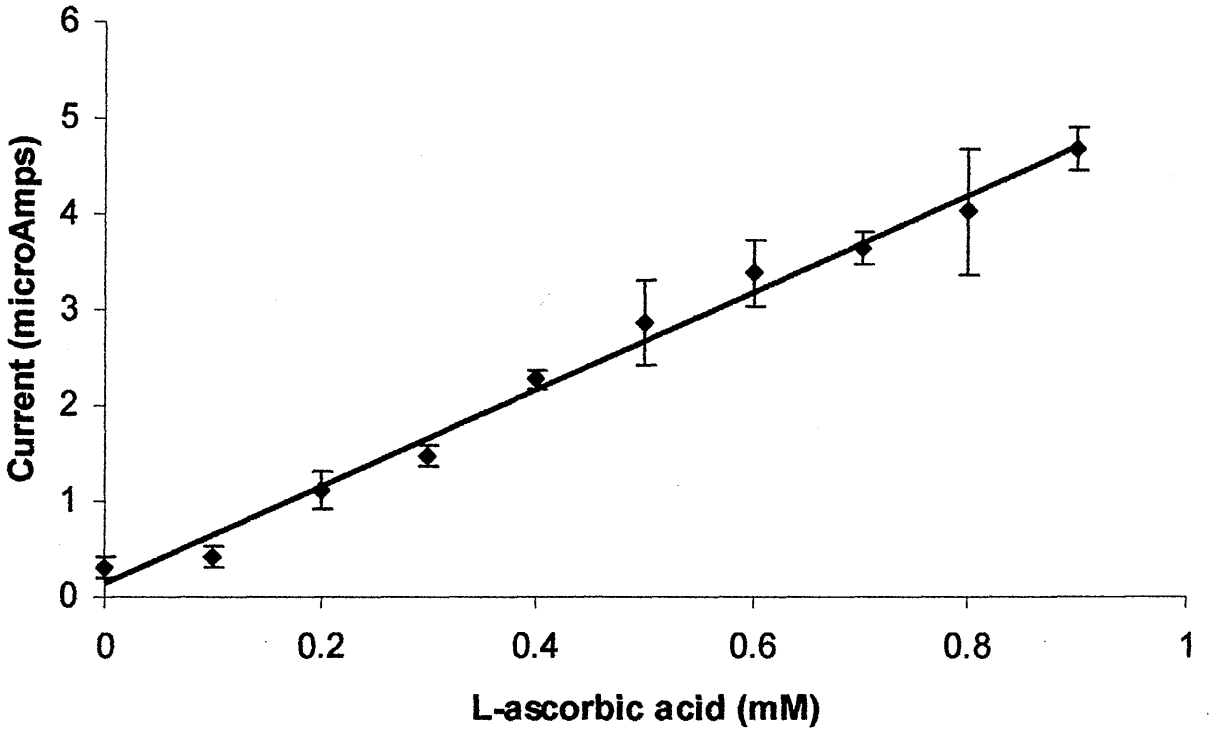


Figure 6.11 Active range of L-ascorbic acid. 500 µl of known concentrations of L-ascorbate acid, in phosphate buffer (100 mM, pH 7), containing 100 mM KCl, was pipetted to the applicator pad, of the enzyme free electrode. The working electrode potential was maintained at +300mV against Ag/AgCl reference electrode. At 150 seconds the current was measured. The experiment was performed in triplicate. 30 units of ascorbate oxidase were used. pH 7 phosphate buffer was employed in the experiment.

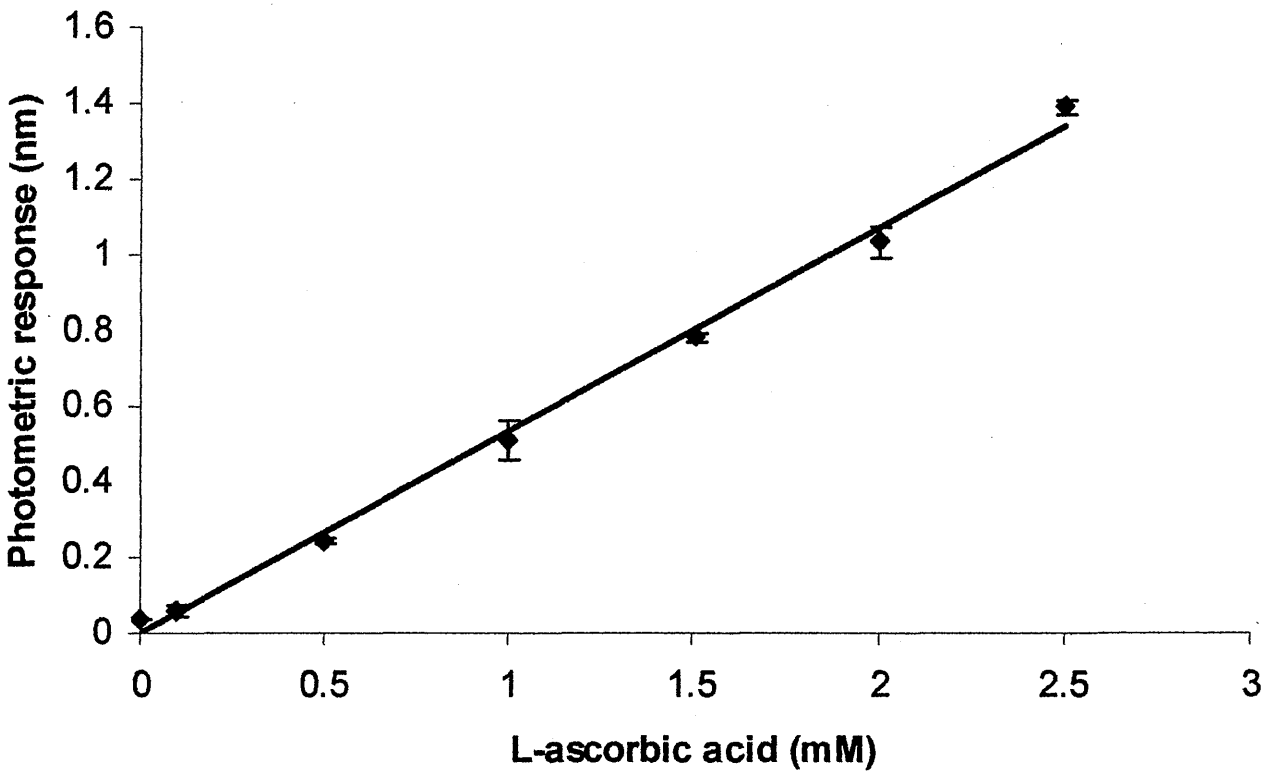


Figure 6.12 Photometric response of L-ascorbic acid using photometric test kit. The absorbance was measured at 340 nm. The samples were tested in triplicates. The method is shown in section 6.2.3.8.

6.3.1.5 Testing with real samples

Horticulture produce is known to contain naturally occurring electroactive substances, which are able to relay "false positives" in biosensor response. One of the most common is ascorbic acid and traditionally a method of reducing this acid is its removal by the use of with the enzyme ascorbate oxidase. This approach cannot be effective in multielectroactive species. To seclude these species, use of a blank electrode was made which was able to measure electroactive species in the absence of immobilised enzymes on the working electrode. The effectiveness of this method is described in (Arif *et al.*, 2001). Utilisation of the blank electrode took place in all subsequent experiments.

6.3.1.6 Investigation of sample preparation

Preparation of the sample was investigated in order to examine the best sensor response in relation to the fruit test kit. Two tomato samples were selected. Three parts of each sample were obtained after a thorough mixing. Each part was treated by a different method. The preparation methods employed included i) centrifugation for 15 minutes (13000 rpm), ii) filtration and iii) a control where the sample was left untreated. In filtration, a portion of the sample was taken by a sterile syringe (10 ml Terumo®, Belgium) and passed through a filter of 0.2 μm. The samples were electrochemically and spectrophotometrically tested for glucose measurement. The results are shown in Figure 6.13. CV values for the test kit were between 0.300-4.932 % and for the sensor 3.917- 16.131 %. The results indicate that processing the sample increased

the sensor response and that filtration was able to give a response with a minimum of error. However centrifugation of the samples led to a more comparable response between sensor and test kit. Nevertheless the controls were not able to demonstrate a high degree of divergence from the test kits. Since the ultimate aim of these biosensors is to use them in field analysis, no processing method was therefore used.

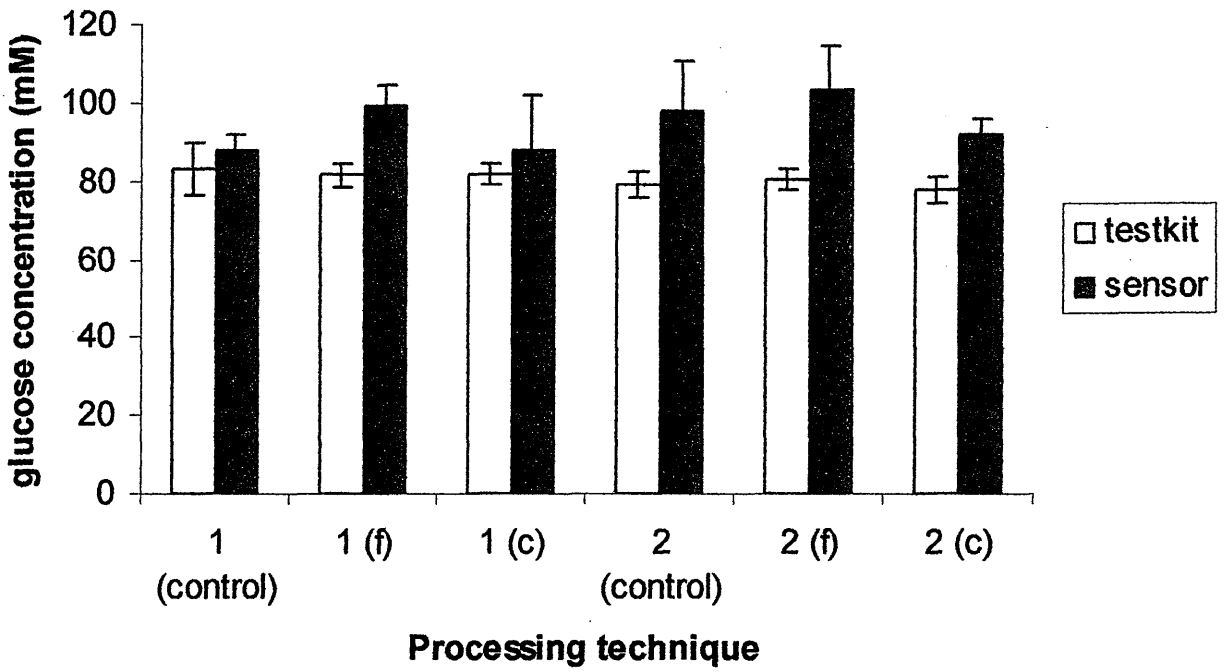


Figure 6.13 Biosensor and test kit measurement of glucose in two tomato samples. The samples were processed by different methologies, where samples f depicts that the sample was filtered before being tested, while c represents that the sample was centrifuged at 13000 rpm. The control is referred to as sample without processing.

6.3.1.7 Testing on real samples

Two varieties of apples namely Bramely and Jonagold varietie and also potatoes were tested for L-glutamic acid and precision of both methods was considered as a measure of reproducibility. It was noted here that precision of the biosensor was as good as the photometric test kit precision. There was only one value below 80 % for the biosensor. The average precision for the biosensor was 90.19 % while for the test kit the average value was 94.00 %. When the L-glutamic acid biosensor was compared directly with the photometric method then the accuracy of only potatoes was above 60 %. This result is noteworthy, because Davies, (1977) reports that L-glutamic acid is one of the most dominant amino acids in potatoes while in apples Elkins *et al.*, (1993) report that asparagine is the predominant acid. One of the limits of the biosensor approach, is that 100 mM of KCl is required in the sample to be tested. To achieve this, sample is usually diluted with the buffer electrolyte. In the case of the L-glutamic acid biosensor the concentration of L-glutamic acid in sample was close to the limit of detection of the biosensor, therefore the accuracy of the biosensor was compromised when the dilution step was taken.

Two types of Russell Burbank potatoes were tested for glucose. These batches were names RB1 and RB2 (depending on the month of delivery). The precision between the potatoes, apples (jonagold and brameley varieties) and tomatoes (miniplum) was above 80 % for the biosensor response (Table 6.2). The average precision of the photometric method was 99.43 % but the average precision of the biosensor was 92.49 %. The precision of the biosensor was considered to be adequate since the biosensor approach is still under development while the test kit approach, has

undergone numerous product evaluations. The accuracy of the biosensor was above 70 % for many of the values. The best accuracy values were observed for Jonagold varieties of apples where the accuracy was above 90 % for three out of four of the values. Generally the accuracy was lowest for the potato samples. Potatoes have been known to contain low concentrations of D-glucose (Burton, 1966), and of the samples tested potatoes contained the lowest D-glucose concentrations. It was considered that possibly the sample matrix may be having an effect on the biosensor response. The results will be discussed in the next section.

Commercial apple, tomato and orange juice was used to measure L-ascorbic acid content in real sample. The average precision of the biosensor was 94.2 %. This compared favourably to the precision of the photometric test kit, which was 94.83 %. Accuracy in this case was also good with the lowest value being 83.26 %.

Measurement of amino acid (Table 6.3) was taken from King Edwards varieties of potatoes and Mini plum tomatoes, and Jonagold varieties of apples. The average value for the precision of the biosensor was 93.04 % and this compared favourably to the ninhydrin method, which had an average precision of 94.54 %. However the accuracy of the biosensor, when compared to the photometric test kit was consistently below 60 %. The limited accuracy of the biosensor in comparison to the ninhydrin method may be a result of the ninhydrin test being not only specific to 'whole' Amino acids but is specific to the detection of amino groups (also measures D-amino acids). This may explain partially the reduced response of the biosensor. Another reason could be due to the specificity of the enzyme for certain amino acids. L-leucine may not be the prominent amino acid present in horticulture produce and

therefore a reduced response was seen. Carangal *et al.*, (1954) states that glutamic acid and aspartic acid are the main amino acids present in tomatoes. The three main amino acids present in potatoes are glutamic acid, glutamine and asparagine (Davies, 1977). While in apples asparagine, aspartic acid, and alanine are the dominant amino acids (Elkins *et al.*, 1993). Kacaniklic *et al.*, (1994) used the amino acid oxidase biosensor to measure the response of the amino acid oxidase to individual amino acids. These responses were compared to the response of the biosensor to phenylalanine. In our case we have used L-leucine as our standared because this gives a response index of 1.13 compared to phenylalanine (Kacaniklic *et al.*, 1994). The response index of the amino acids present in tomato, potato and apples is: glutamic acid (0.29), aspartic acid (0.14), glutamine (0.28), asparagine (0.22), alanine (0.20). The difference in accuracy values between the biosensor and test kits results could be accounted to the ninhydrin method having a higher affinity for the main amino acids in tomatoes, potatoes and apples.

6.3.1.8 Matrix effect:

Of significant concern during the course of this study was the effect of matrix interferents on the sensor performance. The presence of electroactive species in the sample matrix could simply be accounted for by deduction of the background signal from the analyte specific response. However fouling problems could arise due to the presence of solid materials in the matrix that could adsorb onto the working electrode surface and hence reduce the effective working area of the electrode,

Table 6.1 glutamate biosensor results comparing the accuracy and precision of the biosensor with the established test kit method. The number in brackets is the analyte value in mM.

Sample	Accuracy [a] (%)	Precision [b] of Biosensor (%)	Precision [b] of test kit (%)
Potato	73.20	90.34 (0.17)	95.38 (0.23)
2	67.67	92.5 (0.13)	98.5 (0.20)
3	67.47	94.93 (0.29)	70.95 (0.19)
4	75.09	92.03 (0.33)	98.66 (0.44)
Apple (Jonagold)	< 60	93.96 (0.089)	96.77 (0.17)
2	< 60	87.16 (0.095)	88.78 (0.14)
3	< 60	90.99 (0.076)	100 (0.13)
4	< 60	98.89 (0.088)	95.95 (0.12)
Apple (bramely)	< 60	74.06 (0.0012)	95.91 (0.054)
2	< 60	96.52 (0.009)	99.41 (0.026)
3	< 60	86.62 (0.0016)	98.35 (0.086)
4	< 60	84.33 (0.0035)	89.29 (0.063)

[A] $$\text{Accuracy} = 100 - \left[\frac{[\text{biosensor sample}] - [\text{photometric sample}]}{[\text{photometric sample}]} \times 100 \right]$$

[B] $$\text{Precision} = 100 - \left[\frac{(\text{SD of biosensor sample}) \text{ or } (\text{SD photometric sample})}{\text{Mean value of biosensor or photometric (sample)}} \times 100 \right]$$

Table 6.2 Glucose biosensor results comparing the accuracy and precision of the biosensor with the established test kit method. The number in brackets is the analyte value in mM.

Sample	Accuracy	Precision of Biosensor (%)	Precision of test kit (%)
Potato (rb1)	78.508	93.78 (9.66)	99.265 (12.30)
2	70.921	87.233 (9.67)	99.368 (13.63)
3	62.357	91.084 (6.03)	99.769 (9.68)
4	70.155	91.422 (9.57)	98.898 (13.64)
Potato (RB2)	78.417	92.848 (18.75)	98.425 (23.91)
2	70.855	86.637 (12.05)	99.536 (17.01)
3	< 60	92.223 (8.49)	99.273 (21.53)
4	< 60	88.204 (9.28)	99.394 (22.91)
Apple (Jonagold)	< 60	94.396 (57.69)	99.892 (105.36)
2	95.68	95.056 (101.93)	99.8918 (97.71)
3	93.606	95.403 (125.17)	99.465 (117.64)
4	98.385	96.27 (117.41)	99.61 (115.55)
	93.595	91.377 (109.16)	99.539 (116.63)
Tomato (miniplum)	70.136	95.611 (61.36)	99.882 (87.48)
2	83.979	96.406 (74.48)	99.598 (88.69)
3	78.044	95.312 (99.42)	99.028 (81.52)
4	71.905	89.035 (103.23)	99.54 (80.59)

Table 6.3 L-ascorbic acid biosensor results comparing the accuracy and precision of the biosensor with the established test kit method. The number in brackets is the analyte value in mM.

Sample	Accuracy (%)	Precision of Biosensor (%)	Precision of test kit (%)
Tomato	83.26	93.62 (0.17)	97.6 (0.21)
2	85.23	87.68 (0.15)	98.62 (0.18)
3	91.49	95.63 (0.24)	95.36 (0.26)
Apple	95.26	98.39 (0.08)	89.36 (0.09)
2	94.71	94.26 (0.10)	93.45 (0.11)
3	93.61	96.82 (0.07)	94.35 (0.08)
Orange	97.39	97.15 (0.31)	96.23 (0.32)
2	97.51	92.52 (0.36)	91.23 (0.37)
3	98.79	91.83 (0.36)	97.23 (0.36)

Table 6.4 L-amino acid biosensor results comparing the accuracy and precision of the biosensor with the established photometric method. The number in brackets is the analyte value in mM.

Sample	Accuracy (%)	Precision of Biosensor (%)	Precision of test kit (%)
Potato (King Edwards)	< 60	94.57 (3.24)	94.5 (63.35)
2	< 60	88.26 (3.54)	99.87 (68.35)
3	< 60	89.51 (3.79)	99.11 (60.74)
4	< 60	95.33 (3.63)	97.52 (70.68)
Apple (Jonagold)	< 60	94.89 (1.25)	90.39 (5.32)
2	< 60	94.1 (1.16)	92.62 (4.32)
3	< 60	93.78 (2.12)	92.78 (4.93)
4	< 60	91.67 (4.93)	94.86 (1.815)
Tomato (miniplum)	< 60	96.06 (2.10)	96.71 (30.12)
2	< 60	88.03 (1.92)	93.7 (8.82)
3	< 60	95.85 (1.41)	87.58 (25.20)
4	< 60	94.44 (1.21)	94.78 (28.98)

resulting in a lower analyte-generated current than expected or higher variation in the signal achieved. The effect of sample preparation on the biosensor and the test kit measurement of glucose and Amino acids were investigated.

Glucose and L-amino acid biosensors were tested on fruit samples inoculated with glucose or leucine standard solutions, respectively. Figure 6.13 is able to show a comparison between spiked with 1 mM of L-leucine and non- spiked solutions. The graph demonstrated the enzyme high Km for L-leucine. Low accuracy values for 'real' samples demonstrated the possibility of low levels of L-leucine in samples. The possibility of the enzyme amino acid oxidase being inhibited was improbable, since a high response was achieved in spiked solutions.

The matrix effect was investigated further with the glucose biosensor (Figure 6.14). In this case 3 mM of glucose was used to spike the solution. Glucose levels in spiked solutions were understandably higher then non-spiked solutions and glucose levels of up to 200 mM in non-spiked solutions concluded that the fruit matrix had no obvious effect on glucose oxidase.

6.4 Conclusion

The L-ascorbate oxidase biosensor was able to detect ascorbate concentrations up to 0.6 mM. The biosensor showed good reproducibility. The optimum potential for the biosensor was 0.2 mV against Ag/AgCl. 15 units of enzyme was considered to be the optimum enzyme for the biosensor.

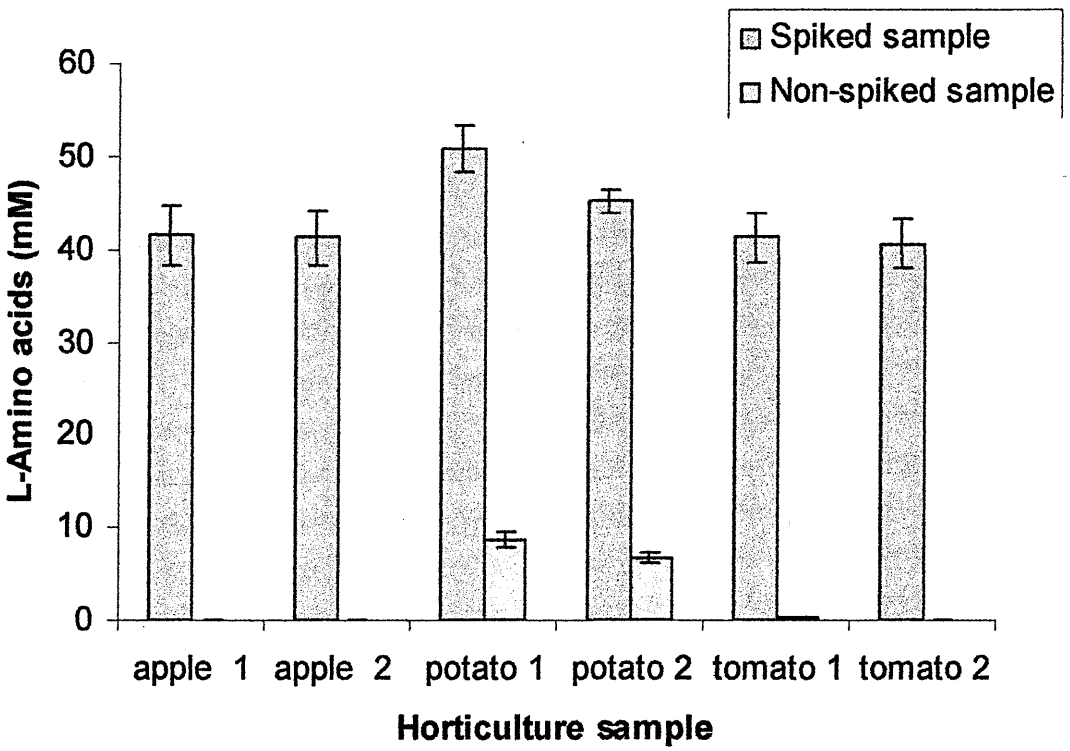


Figure 6.14 L-amino acid biosensor was used to measure L-amino acid content in apples, potatoes, and tomatoes. Samples were initially spiked with 40 mM of L-leucine (blue) and these were compared with control samples containing no additional L-leucine (purple). The method procedure was completed by deposition of 40 µl sample (spiked or non spiked) in 100 mM phosphate buffer (pH 7.4, 100 mM KCl), onto the applicator pad and the working electrode poised at a potential of +350 mV versus the Ag/AgCl reference. The system was allowed to equilibrate in the presence of sample. The samples were tested in triplicate.

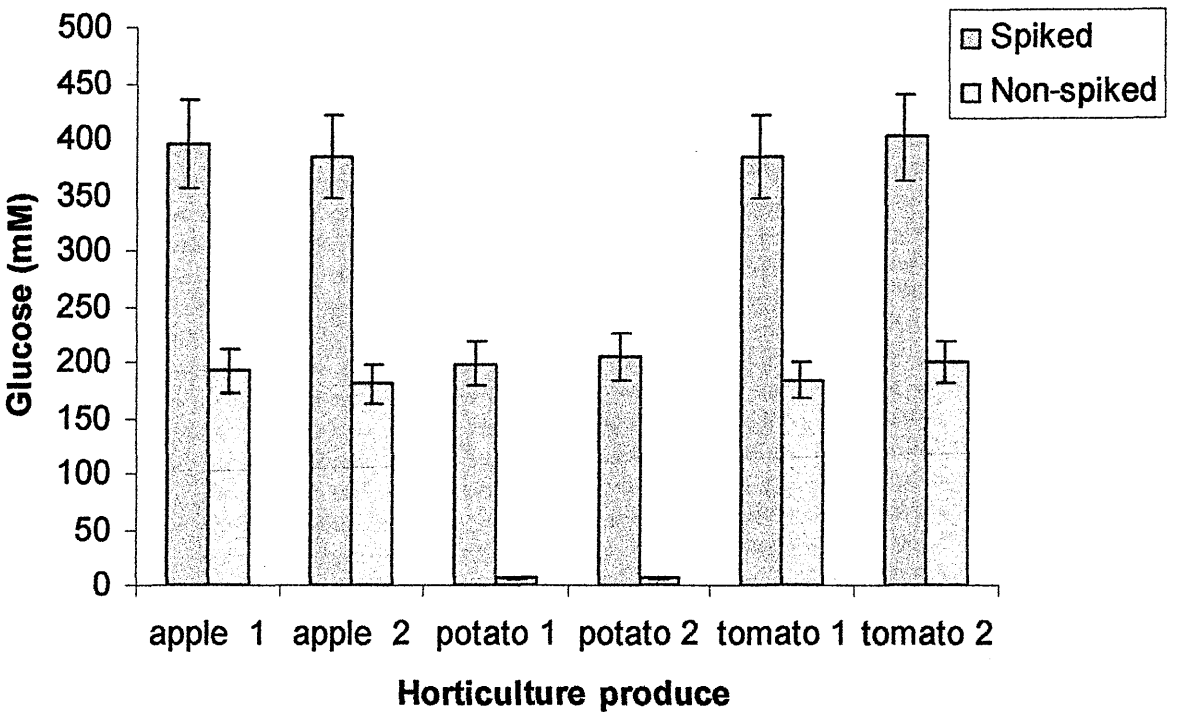


Figure 6.15 D-glucose biosensor was used to measure glucose content in apples, potatoes, and tomatoes. Samples were initially spiked with 200 mM of D-glucose (blue) and these were compared with control samples containing no additional D-glucose (purple). A 30 µl volume of buffer (phosphate buffer (100 mM) pH 7.4 containing 100 mM KCl) was deposited onto the applicator pad and the working electrode poised at a potential of 350 mV versus the Ag/AgCl reference. After 110 seconds equilibration time, 30 ml of sample (D-glucose spiked or non spiked) was added. Readings were taken 240 seconds after the addition of the sample. The samples were tested in triplicate.

The correlation results between the standard analytical methods (test kits) and the biosensors are very encouraging, and indicate the validity of the biosensor approach for monitoring these analytes in horticultural produce samples. As would be expected, the sensors did not outperform the standard test kits, but have not been subjected to the many years of optimisation that the kits have been subjected too and key operational parameters, such as ease-of-use, speed, cost and field-based usage are encompassed by the biosensor approach. When considering the biosensor devices themselves, the glucose biosensor provided a significantly higher degree of analytical performance (detection limit, assay range, accuracy and precision) than the glutamic acid and amino acid sensors. The amino acid sensor was found to consistently underestimate the amino acid content in the samples. It was postulated that this was due to the amino acids found in the horticultural samples having lower affinities to the L-amino acid oxidase enzyme than those amino acids (L-leucine, or L-phenylalanine) used to calibrate the amino acid biosensor.

7 DEVELOPMENT OF A MULTISENSOR ARRAY FOR THE MEASUREMENT OF L-ASCORBIC ACID, L-MALIC ACID AND D-GLUCOSE

In the previous Chapters, biosensors were developed for the detection of L-ascorbic acid, L-malic acid and D-glucose. One of the disadvantages of these sensors is that they are only able to detect single analytes at one time. The development of sensor array of these enzyme electrodes will be advantages for the assessment of horticulture produce quality, since several analytes can be analysed at the same time. A screen-printing approach has been used in this case to develop a sensor devise that is able to detect L-ascorbic acid, L-malic acid and D-glucose simultaneously.

7.1 Methods

7.1.1 Multisensor array fabrication

Screen-printed sensors were fabricated in house in large batches employing a multi-stage screen-printing process by use of a DEK 248 printer (DEK, Weymouth, UK). These electrodes consisted of carbon (rhodensied) working and counter (graphite) electrodes and a Silver/Silver Chloride (Ag/AgCl) reference electrode. These were produced using screens with the appropriate masking stencil designs. The electrodes were printed sequentially with a single deposit and were defined with a final insulating shroud as illustrated in Figure 7.1. The multisensor array consisted of a separate counter and reference electrode for each one of the four working electrodes.

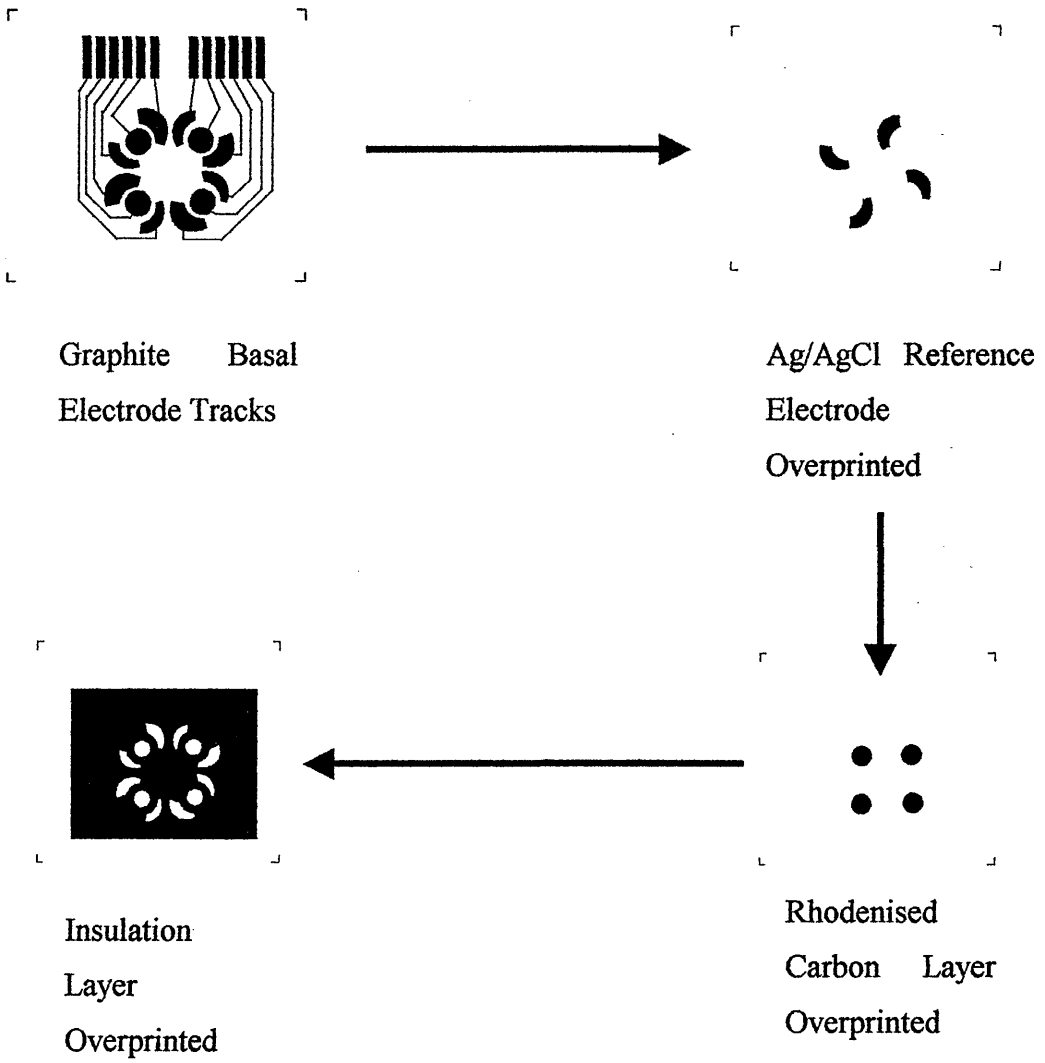


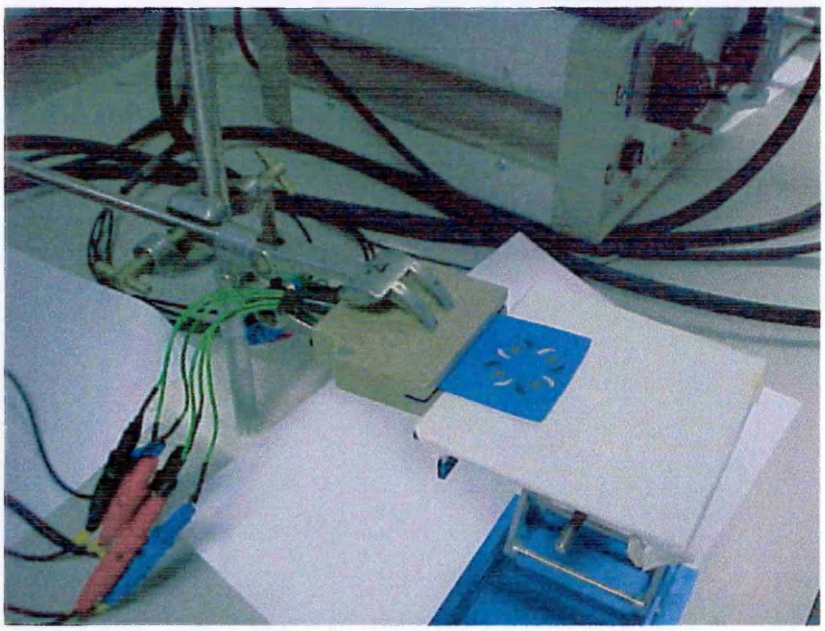
Figure 7.1 The screen printing process in sensor fabrication.

The sensors were printed on to 250 μm thick 'Melinex' (Cadillac Plastic, Swinton, UK) polyester sheets (29 m x 305 mm). This enabled each screen to print 48 multisensors arrays per sheet. The printer parameters were set to print at a 4 psi squeegee pressure at a 50 mm sec^{-1} carriage speed with a 2.5 mm print gap. First a layer of basal graphite tracks was printed with MCA 145R carbon ink, forming the basic working and counter electrodes. This was dried and followed by screen printing a layer of rhodenised carbon ink on to the working electrode surface. The electrodes were dried and were followed by a pad of reference electrode ink consisting of 15 % silver chloride in silver paste. The electrodes were then uniformly defined and the basal tracks insulated with a blue layer of 242-SB epoxy-resin based protective coating ink, cured by heat treatment at 125 °C for two hours. This resulted in a 6 mm diameter working electrode, giving a planer area of 28 mm^2.

7.1.2 Instrumentation Development

In order to connect the screen printed electrode to the potentiostat it was necessary to develop a flexible and robust connection system that is compatible with the electrode terminals. Connection leads were constructed in house to connect the multisensor array with consistently reliable electrical integrity. The connector module was combination of Personal Computer (PC) connector module connected to the leads (Figure 7.2).

A)



B)

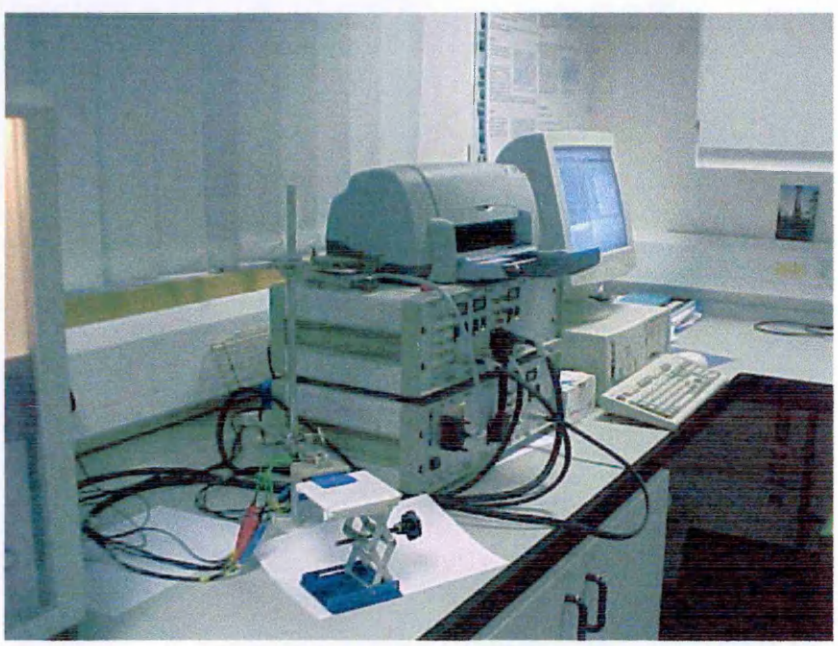


Figure 7.2 a) Screen printed electrode array connecter module. B) Photograph of full 'autolab'working environment.

7.1.3 Multisensor array measurement

7.1.3.1 Immobilisation of Enzymes

Glucose oxidase (1.842 µl) and L-ascorbic acid oxidase (10 µl) were immobilised onto separate working electrodes of the multiarray sensor according to the method described in Chapter 6. In the case of malic enzyme for the measurement of L-malic acid, 10 µl of the enzyme was immobilised onto the working electrode of the multisensor array, the method was identical to that described in Chapter 4. The fourth working electrode was left as a blank. The multisensor electrode (containing immobilised enzymes), was then air dried for 3 hours.

7.1.3.2 Test procedure

Cotton mesh was soaked in 10 ml of electrolyte buffer (phosphate buffer pH 7, containing 100 mM KCl). This was inserted onto the screen printed electrode. 500 µl of known concentrations of sample (in electrolyte buffer), was pipetted onto the applicator pad. The working electrode potential was maintained at +300mV against the Ag/AgCl reference electrode. The system was allowed to equilibrate in the presence of sample and the current value noted after 150 seconds.

7.2 Results and discussion

7.2.1 Principle of the multisensor array

The multisensor array contains four separate working electrodes. Each working electrode encloses a separate counter and reference electrode. Glucose oxidase, L-

ascorbate oxidase and malic enzyme were immobilised onto three of the working electrodes. The fourth working electrode had been left blank and thus measured the background current. The principle of the multisensor array is best illustrated in Figure 7.3.

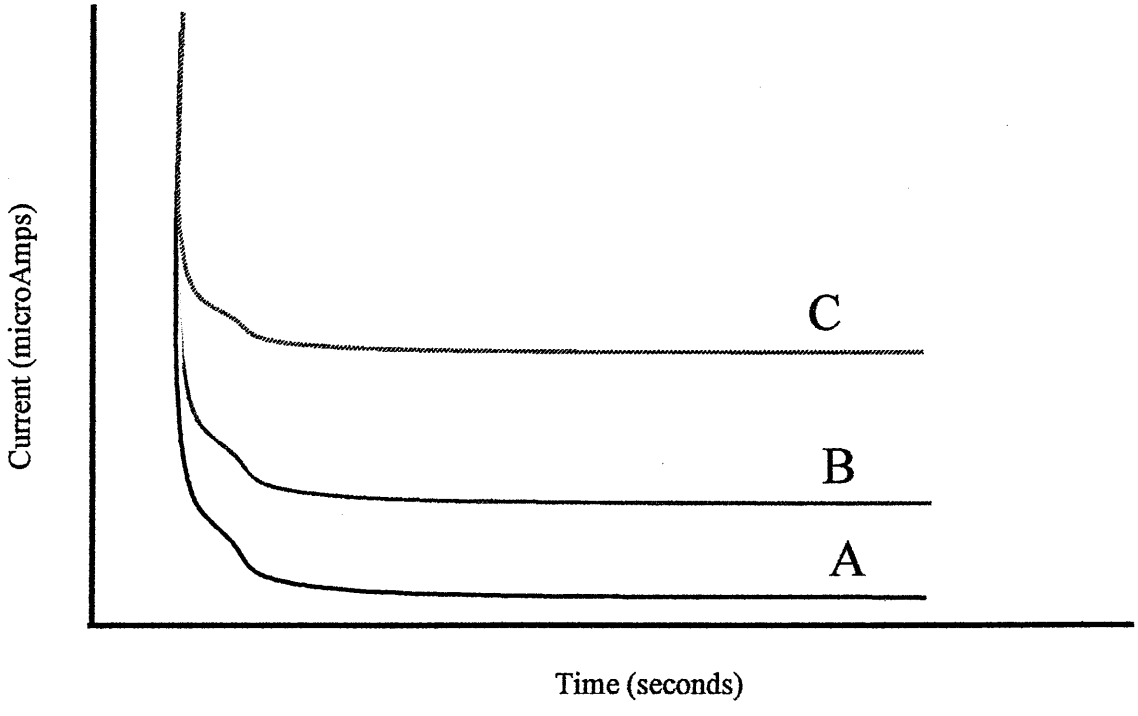


Figure 7.3 Diagramatic view describing principle of multisensor array. Where A represents the background current, B represents free L-ascorbic acid + A, and C correspond to Analyte signal (glucose or L-malic acid) + B.

As the sample is added to the applicator pad of the multisensor array assembly, there was a change in current, and the signal was equilibrated over time (Figure 7.3). The multisensor was able to give four signals. For the sake of simplicity, only three

signals are shown in Figure 7.3 where C (black dotted line) represents signal from L-malic acid or D-glucose detection. The blue line represents the signal gained by the ascorbate oxidase immobilised electrode (background current A). The enzyme was able to convert L-ascorbic acid to dehydroascorbic acid. The red dotted line represents the signal of the enzyme free electrode. This value represents total L-ascorbic acid in sample and also the background current (B). The doted black line represents the signal for the production of NADPH (L-malic acid detection) or H_2O_2 (D-glucose detection). For the sake of simplicity only one of the two signals was shown in Figure 7.3. This signal (C) represents the background current as well as free L-ascorbic acid in sample and also the current generated from the analyte of interest (D-glucose response or L-malic acid response). Response for L-malic acid or D glucose present in the sample was determined by subtracting different C values from signal B. Determination of L-ascorbic acid was achieved by subtracting signal B from A.

To determine the effects of cross-talk between analyte responses, the multianalyte biosensor was tested individually for each of the analytes (where only one enzyme was immobilised on the multienzyme biosensor). The sensor was also tested where all three analytes were measured on the same pad.

7.2.2 L-malic acid

A linear relationship between L-malic acid concentration (x) and current response (y) was measured up to 0.5 mM. The equation of the line was $y = 1.1926x + 0.0227$.

The limit of detection for L-malic acid, calculated as 2.5 × the standard deviation (SD) of the zero analyte response was 0.042 mM (Figure 7.4).

When the malate biosensor was tested simultaneously with other analytes the equation of the line was y = 1.3459x + 0.0131 with the correlation coefficient (r) 0.9915. The limit of detection was 0.044 mM (Figure 7.5).

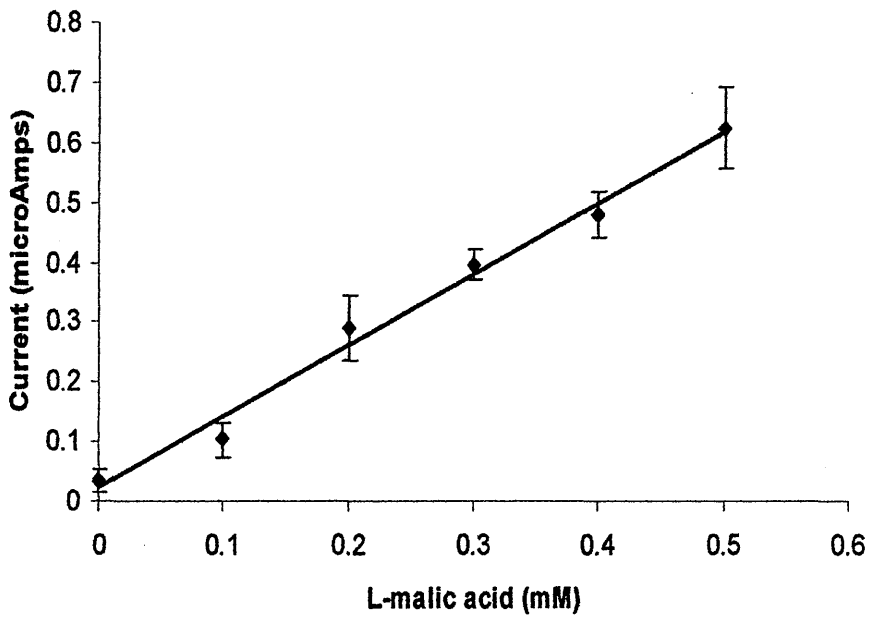


Figure 7.4 L-malic acid was measured individually from 0-0.5 mM of L-malic acid. The test was done in triplicate, at a potential of 300 mV against Ag/AgCl. 100 mM Phosphate buffer PH 7 containing 100 mM of KCl was used.

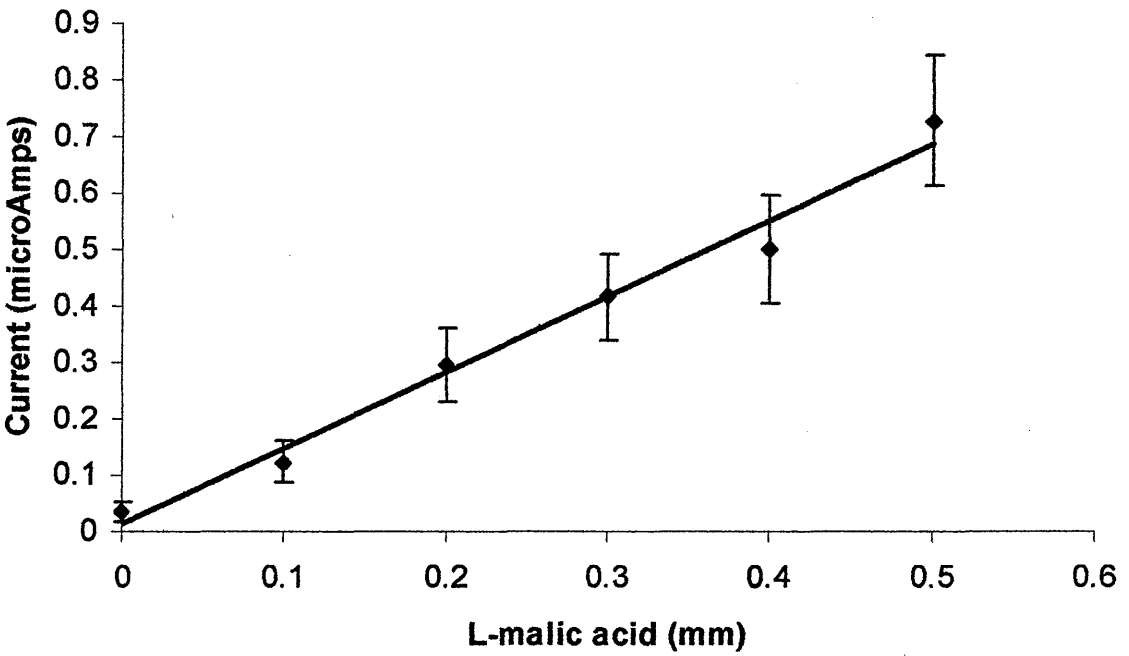


Figure 7.5 L-malic acid was measured using the multiarray format. A linear range from 0-0.5 mM of L-malic acid was constructed. The test was done in triplicate, at a potential of 300 mV against Ag/AgCl. pH 7 phosphate buffer (containing 100 mM of KCl) containing L-malic acid, L-ascorbic acid and D-glucose was used.

7.2.3 D-glucose

When the biosensor was tested individually for D-glucose the linear range was measured up to 5 mM. The equation of the line was $y = 0.7174x + 0.0889$ while the correlation coefficient was 0.9995. The limit of detection was observed as 0.035 mM (Figure 7.6).

During multianalyte testing of the biosensor the equation of the line was y = 0.6754x + 0.1003, the correlation coefficient was 0.9996. The limit of detection was 0.031 mM (Figure 7.7).

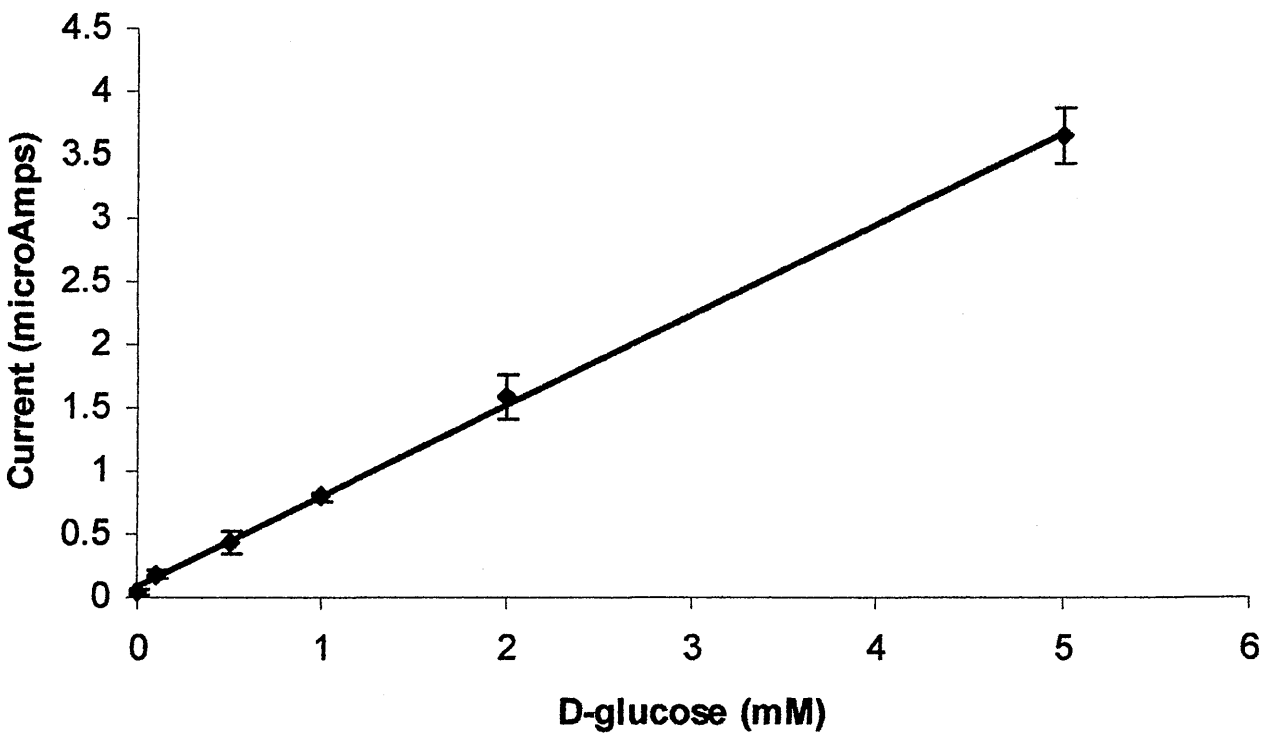


Figure 7.6 D-glucose was measured individually from 0-5 mM. The test was done in triplicate, at a potential of 300 mV against Ag/AgCl. 100 mM Phosphate buffer pH 7 containing 100 mM of KCl was used.

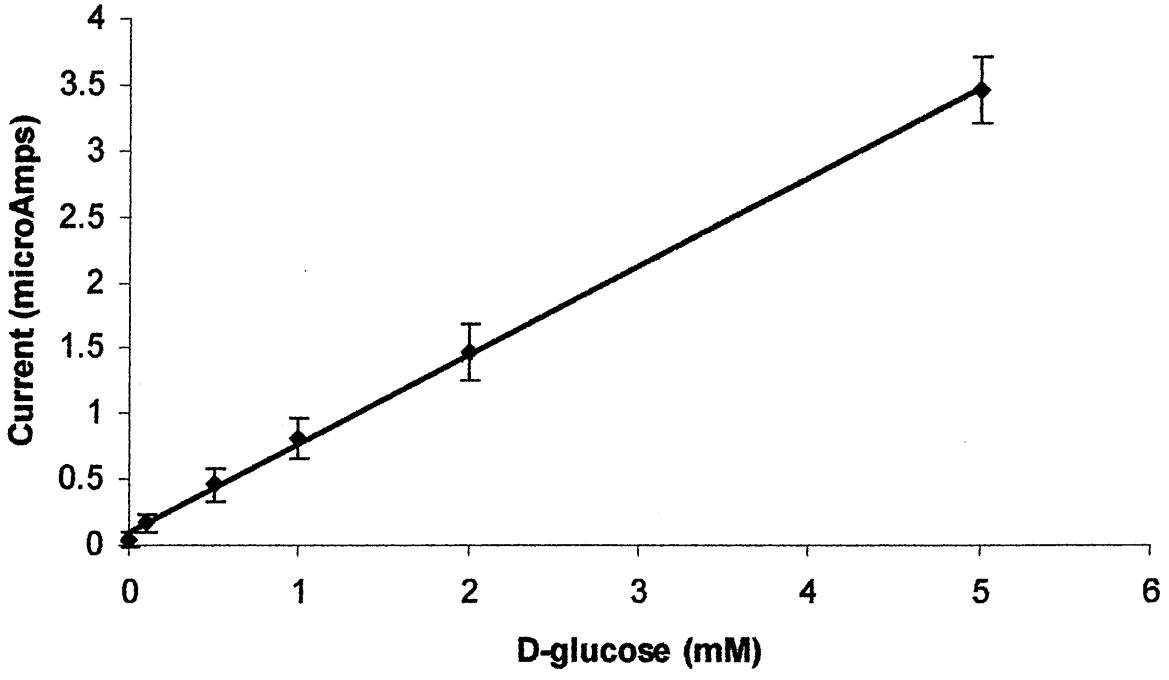


Figure 7.7 D-glucose was measured using the multiarray format. A linear range from 0-5 mM of D-glucose was constructed. The test was done in triplicate, at a potential of 300 mV against Ag/AgCl. pH 7 phosphate buffer (containing 100 mM of KCl) containing L-malic acid, L-ascorbic acid and D-glucose was used.

7.2.3.1 L-ascorbic acid

Free L-ascorbic acid was measured on the enzyme free electrode; a linear range up to 0.5 mM was measured. The equation of the line was $y = 5.52x$ while the correlation coefficient was 0.9938. The limit of detection of the sensor was 0.016 mM (Figure 7.8).

When L-ascorbic acid was measured with other analytes, the equation of the line was $y = 5.6471x$ while the correlation coefficient was 0.9932. The limit of detection of the sensor was 0.017 mM (Figure 7.9).

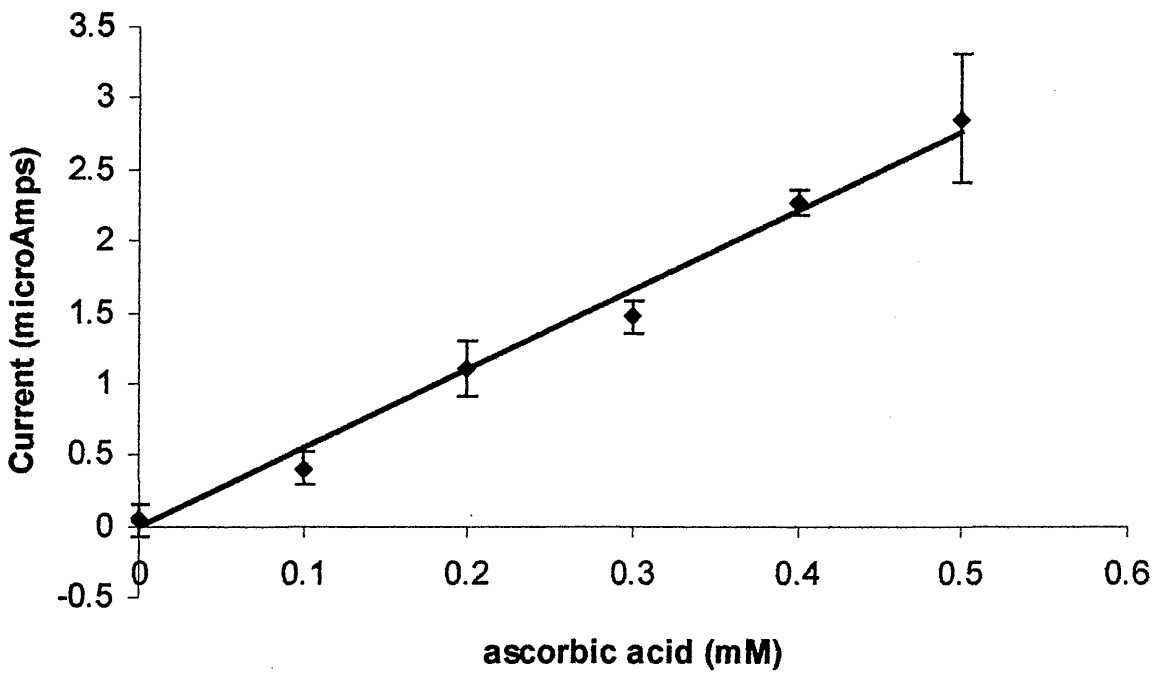


Figure 7.8 L-ascorbic acid was measured individually from 0-5 mM. The test was done in triplicate, at a potential of 300 mV against Ag/AgCl. 100 mM Phosphate buffer pH 7 containing 100 mM of KCl was used.

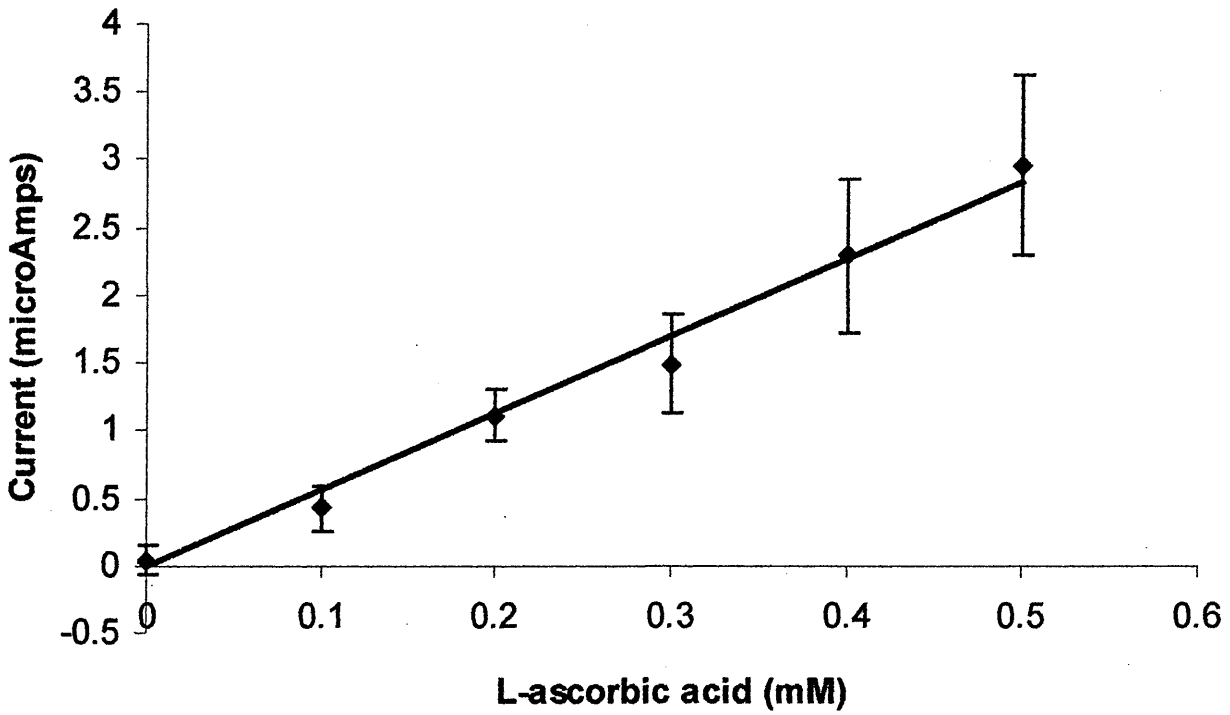


Figure 7.9 L-ascorbic acid was measured using the multiarray format. A linear range from 0-0.5 mM of L-ascorbic acid was constructed. The test was done in triplicate, at a potential of 300 mV against Ag/AgCl. pH 7 phosphate buffer (containing 100 mM of KCl) containing L-malic acid, L-ascorbic acid and D-glucose was used.

7.2.4 Comparison of both methods

During the function of the multianalyte biosensor four electroactive compounds are produced. Therefore there is a possibility that cross-reactivity of these products is possible. Volotovsky and Kim (1998) have suggested that since the enzymes have not been co-immobilised on the same working electrode, therefore the electroactive product produced would be diluted in the ambient media and therefore the effect on

the net signal would be minimal. In this multiarray biosensor a non-enzyme electrode has been included so that is able to measure not only free L-ascorbic acid but also any electroactive products such as hydrogen peroxide, which may have diffused. Individual testing of each of the analytes was taken as well as analysing the multisensor with all three enzymes immobilised together. The results of the accuracy and precision of the multisensor with respect to testing individual analytes is shown in tables 7.1,7.2,7.3. The results show that the precision of the multianalyte biosensor ranged from 71% to almost 85%. This was in close correlation to the malic enzyme biosensors only measuring L-malic acid (range 72 % to 89 %). The accuracy of the multisensor with respect to L-malic acid was above 90%. In the case of the glucose sensor the precision was 60 % for 0.1 mM of D-glucose, nevertheless the precision for all other concentrations was above 70% and compared favourably to the precision of the single analyte testing of D-glucose. The accuracy of the multiarray was above 80 % in all cases.

Measurement off free L-ascorbic acid has shown that the precision for 0.1 mM of L-ascorbic acid was low while all other precision values were higher then 75 %. The accuracy of the L-ascorbic acid multiarray sensor was above 75% in all cases.

These results are able to demonstrate that the effect of interference species on the multiarray biosensor may actually be limited. When measuring more then one analyte the accuracy and precision of the multiarray may be slightly lower then when measuring single analytes but this disadvantage may be overcome with the benefits of measuring more then one analyte simultaneously, and thereby saving time and money.

Table 7.1 Precision and accuracy values for L-malic acid where it was tested individually on the multiarray pad and also tested along with L-ascorbic acid and D-glucose using the array sensor. The results are shown in triplicate. The value in brackets is the amount of analyte in mM.

L-malic acid (mM)	Precision %		Accuracy (%)
	Single	Multianalyte	
0.1	72.54 (0.110)	71.71 (0.089)	80.65
0.2	81.52 (0.192)	78.49 (0.188)	97.81
0.3	93.59 (0.321)	81.58 (0.304)	94.77
0.4	91.85 (0.442)	80.97 (0.421)	95.35
0.5	89.34 (0.511)	84.14 (0.426)	83.46

Table 7.2 Precision and accuracy values for D-glucose where it was tested individually on the multiarray pad and also tested along with L-ascorbic acid and L-malic acid using the array sensor. The results are shown in triplicate. The value in brackets is the amount of analyte in mM.

D-glucose (mM)	Precision %		Accuracy (%)
	Single	Multianalyte	
0.1	76.93 (0.122)	60.24 (0.100)	82.37
0.5	80.40 (0.535)	72.88 (0.508)	94.88
1	96.42 (1.056)	80.77 (1.001)	94.81
2	88.96 (2.003)	85.32 (1.846)	92.16
5	94.10 (5.464)	92.57 (4.797)	87.80

Table 7.3 Precision and accuracy values for L-ascorbic acid where it was tested individually on the multiarray pad and also tested along with L-malic acid and D-glucose using the array sensor. The results are shown in triplicate. The value in brackets is the amount of analyte in mM.

Free L-ascorbic acid (mM)	Precision %		Accuracy (%)
	Single	Multianalyte	
0.1	72.40 (0.117)	60.48 (0.111)	95.05
0.2	82.49 (0.236)	82.46 (0.187)	79.43
0.3	92.03 (0.295)	75.64 (0.291)	98.59
0.4	95.84 (0.423)	75.11 (0.317)	75.01
0.5	84.42 (0.510)	77.68 (0.492)	96.39

7.2.4.1 Biosensor cross talk

The multisensor array developed is measuring more then one analyte product simultaneously. Therefore, accuracy of the biosensor could be affected with product cross-talk.

Previous strategies to minimise cross-talk have been mainly membrane based, for example, Mosser *et al.*, (2002) developed a multiarray biosensor for the measurement of glucose, lactate, glutamate, and glutamine. Mosser *et al.*, immobilised oxidase family of enzymes where each one of the biosensors produced hydrogen peroxide as the detectable product. A catalase enzyme membrane was employed over the surface of the immobilised enzymes; this enzyme was rapidly able to remove the hydrogen peroxide produced.

Frebel *et al.*, (1997) was able to simultaneously measure glucose, L-lactate and uric acid with the multisensor array. They were able to reduce cross-talk difficulties by adding a NAFION membrane over the sensor system. Their findings indicated that there was interference from Uric acid, which was able to pass through the membrane. Volotovsky and Kim, (1998) reported a multianalyte biosensor for the determination of glucose, ascorbic acid and citric acid. The enzymes used for glucose and ascorbic acid detection were glucose oxidase and peroxidase. The enzyme peroxidase was used to catalyse ascorbic acid to dehydroascorbate with the reduction of hydrogen peroxide. Since hydrogen peroxide, produced as a result of glucose oxidase catalysed reaction is a substrate for peroxidase, and then there can be considerable cross reactivity in the sample. Volotovsky and Kim, confirm from their findings that there were no cross reactivity problems. They explain, that this is because the enzymes were not coimobolised together but were immobilised on separate working electrodes. The hydrogen peroxide produced was diluted significantly before it reached the peroxidase enzyme.

The work in this thesis has demonstrated an alternative approach in minimising interference and cross reactivity problems. The multisensor array was designed specifically so that all four individual biosensors were circling the centre of gravity of the screen printed electrode. Another feature of the design was that all four individual sensors were in symmetry with each other. The applicator pad (cotton mesh) that was used for this design ensured efficient and rapid sample spreading. These design features ensured even diffusion of liquid sample throughout the multisensor array. Inclusion of a compensator electrode devise (Arif *et al.*, 2001) to

the multi array biosensor ensured that any electroactive substances that were present after dilution, can be measured and then accounted for in the net response for each analyte.

8 CONCLUSIONS

This chapter concludes this thesis with a discussion on the findings of this research. It also identifies future research activities. This chapter aims to achieve the following.

- *To summarise key observations of this research.*
- *To identify the main contributions of this research.*
- *To frame future research activities based on this work.*

8.1 Findings of the Research

This section discusses the key observations of this research.

8.1.1 Determination of key analytes for the measurement of horticulture produce quality

- The need for measurement of horticulture produce quality was evaluated by conducting a literature survey. The survey was carried out on three main types of horticulture produce, namely tomatoes, potatoes and apples. Individual constituents in tomatoes contained 48% sugars and 13% organic acids. During ripening of tomatoes the total amount of glucose in tomatoes increases. The total increase in sugars is affected with the amount of solar radiation the tomato is receiving during ripening. Malic and citric were shown to be the predominant organic acids in tomatoes. Total acidity reaches a maximum with the first colour change (breaker) then decreases past ripeness. In immature fruit there is a higher concentration of malic acid than citric acid but as the fruit ripens, malic

decreases and citric increases or remains level. Glutamic acid is the predominant amino acid in tomatoes. The concentration of glutamic acid increases with ripening.

- Potatoes contain high starch levels. L-malic acid is the second most predominant organic acid in potatoes. L-glutamic acid is one of the major organic acids in potatoes. Studies have shown that there is strong variation in individual and total concentrations of amino acids between varieties and also between years and growing location.
- L-malic acid is the most predominant organic acid in apples. During the maturation of apples the concentration of sugars increase while L-malic acid values decrease. After the apples had been left in storage, a further decrease in malic acid was seen and the possible cause of this was shown to be the increased respiration, which can lead to a decrease in malic acid.

8.1.2 Measurement of horticulture produce quality by conventional means

- The standard laboratory method for liberation of liquid samples in horticulture produce is freeze thawing. This method was not feasible for extraction of liquid samples in the field. Therefore an alternative method where the horticulture produce was grated and pressed was compared with the freeze thawing method. Aubergines, carrots, celery, potatoes and tomatoes were tested with both methods. Freeze thawing liberated maximum liquid sample but this technique required the addition of water or buffer to liberate the liquid sample. Since some

horticulture produce such as potatoes absorb more water then others and hold it in the pulp, an inexact dilution may occur. By grating horticulture produce the sample was broken down into small pieces therefore giving a high surface area when pressed. Therefore, a methodology-using grating followed by pressing is recommended for extracting juice from fresh produce outside the laboratory.

- Various varieties of tomatoes were subjected to a taste panel analysis, which consisted of individuals choosing their favourite varieties in order of preference. The same tomato varieties were then tested in the laboratory for individual analytes and the data was subjected to PCA analysis. Significant differences were observed in the levels of taste components between tomato type and between varieties within types. The components showing these significant differences are citric acid, amino acids, glucose and fructose. However, a high degree of variation was observed between fruit of the same type so good replication will be important to establish differences.
- The statistical analysis was able to show that biosensors have a role in distinguishing between varieties and different types of tomatoes on the basis of taste components. There appears to be a preference for tomatoes within a tight band of citric acid levels (20-26 mM) while the sugar levels varied between tomato types. It should be noted however that overall flavour is a combination of the taste components and the volatile flavour components, the levels of which may also vary between varieties.

8.1.3 Biosensor for the measurement of L-malic acid

Screen-printed electrodes based on malic enzyme with amperometric measurement of NADPH oxidation have proven suitable for the simple, low-cost and rapid analysis of L-malic acid in apple, potato and tomato samples. Addition of mediator is not required since the rhodenised carbon working electrode favoured the oxidation of NADPH at a lower operating potential (+300 mV vs. Ag/AgCl) compared with a number of possible organic acid interferents, excepting L-ascorbic acid. Residual interference effects can be accounted for through use of a compensator electrode. The sensor performance was tested against a photometric kit using real samples, yielding accuracy values within 13.7% of the standard method. Since the measurement process is simple, it is amenable to field-based usage with a minimal training requirement. Low sensor manufacturing costs result in single-use disposable devices, thus negating problems of progressive electrode fouling.

8.1.4 Biosensors for the measurement of D-glucose, L-Amino acids, L-ascorbic acid and L-glutamic acid

- The L-ascorbate oxidase biosensor was able to detect ascorbate concentrations up to 0.6 mM. The biosensor showed good reproducibility. The optimum potential for the biosensor was 0.2 mV against Ag/AgCl. 15 units of enzyme were considered to be the optimum enzyme for the biosensor.
- The correlation results between the standard analytical methods (test kits) and the biosensors are very encouraging and indicates the validity of the biosensor approach for monitoring these analytes in horticultural produce samples. As would be expected, the sensors did not outperform the standard test kits, but have

not been subjected to the many years of optimisation that the kits have been subjected to and key operational parameters, such as ease-of-use, speed, cost and field-based usage are encompassed by the biosensor approach. When considering the biosensor devices themselves, the glucose biosensor provided a significantly higher degree of analytical performance (detection limit, assay range, accuracy and precision) than the glutamic acid and amino acid sensors.

- The amino acid sensor was found to consistently underestimate the amino acid content in the samples. It was postulated that this was due to the amino acids found in the horticultural samples having lower affinities to the L-amino acid oxidase enzyme than those amino acids (L-leucine, L-alanine or L-phenylalanine) used to calibrate the amino acid biosensor.

8.1.5 Multisensor array for the simultaneous detection of D-glucose, L-ascorbic acid and L-malic acid

- Glucose oxidase, L-ascorbate oxidase and malic enzyme, were immobilised on an array multisensor that was able to detect analyte concentrations concurrently at a potential of 300 mV against Ag/AgCl in phosphate buffer pH 7.
- The multisensor array biosensor was at first tested individually with each one of the analytes. The precision of the biosensor was excellent with several values higher then 90%. This biosensor was able to produce triplicate analysis of single analytes in a third of the time of previously developed biosensors.

- The multisensor array was tested for all three analytes simultaneously. The results were compared with the single multisensor array system. Accuracy between both methods was excellent with most values being higher the 90%.

8.2 Future Research

- Chapter three discusses L-malic acid and L-citric acid as being the predominant organic acids in horticulture produce. The ratio of malic acid to citric acid is regarded as significant with regard to flavour acceptance (Hobson & Davies, 1971). There have been a limited number of publications on citric acid biosensors. One of the reasons for this is that, citrate lyase (CL) is the only commercially available enzyme and none of its products are electroactive.

Matsumoto and Tsakatani, (1996) have proposed using a three enzyme method for utilising an electroactive product. This includes utilising C.L. to convert citric acid to oxaloacetate and then converting oxaloacetate to pyruvate by use of oxaloacetate decarboxylase. Hydrogen peroxide was one of the products of pyruvate conversion with the aid of pyruvate oxidase. An alternative methodology could be to convert oxaloacetate with the aid of malate dehydrogenase. Malate dehydrogenase requires NADH as a cofactor. Therefore by measuring NADH the concentration of citric acid could be calculated.

$$\text{Oxaloacetate} + \text{NADH} \xleftrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

One problem that may be envisaged with this method is that the reaction could reverse in some real samples such as apples where L-malic acid is higher then citric acid. This may not be a significant factor since the equilibrium of the oxaloacetate reaction is towards the formation of L-malic acid. Another reason is that pH 7.8 is the optimum for this reaction (Mollering 1985a) while reversal of the reaction takes place at pH 9 or higher (Matsumoto *et al.*, 1996). Therefore this approach could be used in developing citric acid biosensors.

- In Chapter four, taste panel analysis of tomatoes was carried out in six varieties. The results indicate that the panel preferred varieties containing high amounts of sugar but with neutral acidity. Future work could concentrate on measuring other horticulture produce samples for example apples (via taste panel analysis and also measurement of individual analytes with developed biosensors). The large data of results could then be compiled into an Artificial Intelligence system such as neural networks.

- The multianalyte biosensor is able to simultaneously detect a range of important quality indicators present in samples. Individual analytes are then deduced by a series of calculations. Programming the calculation steps into a computer could reduce human error.

- Work could be geared towards the modification of the multianalyte biosensor so that it is able to detect a greater range of analytes simultaneously.
- One of the reasons why the blood glucose biosensor is commercially successful is the stability of glucose oxidase. Further research on the stabilisation of enzyme biosensors could be done. One possibility of stabilising the enzymes could be the combined use of polyelectrolytes such as diethylaminoethyl (DEAE)-dextran, dextran sulphate and Gafquat 755N with polhydroxyl compounds (e.g. lactitol, lactose, maltitol,sucrose). This combination has shown that a high level of stability can be conferred to enzymes in the dehydrated state (Gibson *et al.*, 1996).
- During the time of this thesis, there were no reliable electrochemical analysers that could be used for outside the lab analysis. Quite recently Palmsense (Utrecht, NL) instrument has been developed that can be used to measure key analytes outside of the lab. Therefore future work could be to examine the findings of this work, by analysing analytes in the field.

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FULL PAPER
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L-Malic acid biosensor for field-based evaluation of apple, potato and tomato horticultural produce

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A screen-printed three-electrode amperometric biosensor incorporating malic enzyme for the measurement of L-malic acid in apple, potato and tomato horticultural samples has been developed. The working electrode contained 0.38 mU of immobilised enzyme and was fabricated using rhodinised carbon to facilitate NADPH oxidation at an operating potential of +300 mV *vs.* Ag/AgCl compared with >+600 mV for bare carbon. The linear range of the sensor was 0.028–0.7 mM L-malic acid with relative standard deviations of 3.3–13.3%. When testing with real apple, potato and tomato samples, the sensor accuracy was within 13.7% of a standard commercially available photometric test kit. The sensor approach is cheap, simple to perform and rapid (6 min), requiring only buffer–electrolyte and a small sample volume.

Introduction

The selection of a particular foodstuff by a consumer is largely based on sensory perceptions with taste, which is influenced by diverse factors including saltiness, sweetness, bitterness and acidity as perhaps the most important factors.[1] Texture is also a key parameter and is dictated by many factors including moisture content and fat, carbohydrate and protein levels.[2] Other important sensory factors include the aroma, shape and colour of the foodstuff.[1]

L-Malic acid and citric acid are major organic acids in most fruits and vegetables. Organic acids contribute greatly to taste, particularly of fruit, with a balance of sugar and acid giving rise to the desirable taste of specific produce.[3] Significant increases in L-malic acid concentration have been shown to serve as a primary indicator of fruit maturity.[4,5] Hence measurement of L-malic acid provides a more objective means of determining the ripeness and hence 'shelf life' of horticultural produce than simple appearance and taste.

Current methods for the determination of L-malic acid in horticultural produce include both liquid and gas chromatography, with the latter method being less widely employed owing to difficulties in isolating and derivatising fruit acids.[6,7] Alternative methods include capillary isotachophoresis[8] and sequential injection Fourier transform infrared (FTIR) spectrometry.[9] Commercially available test kits for L-malic acid also exist, based on enzymatic assay with photometric detection.[10]

Biosensors, incorporating L-malic acid-specific enzymes coupled to electrochemical transducers have also been developed. Benefits include simplicity, rapidity, economy, portability and minimal sample size. Two enzyme types have been used, malic enzyme (ME) [malate dehydrogenase (decarboxylating) (NADP)] (EC 1.1.1.40) and malate dehydrogenase (MDH) (EC 1.1.1.37), the latter catalysing the reaction:

$$\text{L-malic acid} + \text{NAD}^+ \overset{\text{MDH}}{\rightleftharpoons} \text{oxaloacetic acid} + \text{NADH} + \text{H}^+$$

Generally, the reduced NADH co-factor is measured amperometrically and related stoichiometrically to L-malic acid levels. Owing to the low equilibrium constant (k_{eq} = 6.4 × 10^{-13} M), hydrazine, which reacts with the carbonyl group in oxaloacetate, has been used to promote the forward reaction.[11] A similar approach using glutamate–oxaloacetate transaminase has also been reported.[12] An alternative approach uses an NADH oxidase (diaphorase) to regenerate NAD^+ with O_2 consumption measured with a Clark oxygen electrode.[13] Other strategies include the use of oxaloacetate decarboxylase and pyruvate oxidase to convert oxaloacetate into acetyl phosphate *via* pyruvate with O_2 consumption again measured with an oxygen electrode.[14] Our method uses malic enzyme (ME), which has a k_{eq} of 5.1 × 10^{-2} M, thus negating the requirement for additional reagents to promote the forward reaction:

$$\text{L-malic acid} + \text{NADP}^+ \overset{\text{ME}}{\rightleftharpoons} \text{pyruvic acid} + \text{NADPH} + \text{CO}_2 + \text{H}^+$$

Gajovic *et al.*[15] reported the coupling of ME to salicylate hydroxylase, resulting in the regeneration of $NADP^+$ and L-malic acid determination *via* electrochemical measurement of oxygen consumption. Owing to $NADP^+$ recycling, less then 0.025 mM of co-factor was required. Messia *et al.*[16] used pyruvate oxidase in association with ME with amperometric determination of the hydrogen peroxide by-product at +650 mV *vs.* an Ag/AgCl reference electrode. Matsumoto *et al.*[17] recommended ME over MDH owing to a preferred pH optimum of 7.8 compared with 9.5 for MDH.

The presence of electroactive interferents in real samples has led to the widespread coupling of mediators to enzymes in biosensor applications to allow the use of lower detection potentials.[18] An alternative approach uses electrocatalysts to selectively decrease the oxidation potential of the target analyte. We have evaluated a large number of commercially available metallised carbons and identified a screen-printable rhodinised carbon with excellent electrocatalytic and enzyme immobilisation properties.[19–22]

The aim of this work was to develop stable, cheap, single-use (disposable) screen-printed electrochemical sensors for the rapid and reproducible measurement of L-malic acid in apple, potato and tomato horticultural preparations. Such an approach would provide a simple, rapid, field-based tool for determining the optimum time to harvest produce with respect to the key parameters of ripeness and taste. Simplicity of the measurement process was paramount, owing to the intended field-based usage of the device and target end-user. Consequently, electrocatalytic rhodinised carbon was used in this study to allow the non-mediated detection of NADPH at low potentials, an approach

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that is of interest for the general exploitation of dehydrogenases in non-mediated biosensor applications. The performance of the device was compared against a standard photometric procedure and tested with real horticultural samples.

Experimental

Reagents

The buffer used was NaH_2PO_4–Na_2HPO_4, pH 7.4 (100 mM unless stated otherwise). Buffer–electrolyte solutions also contained 0.1 M KCl. L-Malic acid, L-tartaric acid, L-citric acid, L-glutamic acid, NADP and NADPH were obtained from Sigma-Aldrich (Poole, Dorset, UK) and prepared in buffer–electrolyte as required. Malic enzyme (malate dehydrogenase, EC 1.1.1.40), from chicken liver (specific activity 19.6 units mg^{-1}) and hydroxyethylcellulose (HEC) were purchased from Fluka (Buchs, Switzerland). Solutions were prepared in de-ionised reverse osmosis water (Elgastat System, Elga, High Wycombe, UK).

Sensor fabrication

Three-electrode devices were mass-manufactured in-house by a multi-stage screen-printing process using a DEK 248 machine (DEK, Weymouth, UK) and screens with appropriate stencil designs (60 per screen) fabricated by DEK Precision Screen Division. The stainless-steel screen mesh was mounted at 45° to the print stroke with 77 wires cm^{-1} and emulsion thickness of 13 and 18 μm for the solvent and water-resistant screens, respectively.

Devices were printed on to 250 μm thick polyester sheet (Cadillac Plastic, Swindon, UK). The circular electrocatalytic working electrode (planar area 0.16 cm^2) was fabricated from MCA 4a (MCA Services, Cambridge, UK), a commercially available carbon powder containing 5% rhodium plus promoters, made into a screen-printable paste by mixing 1:4 in 2.5% w/v HEC in buffer–electrolyte. The reference electrode ink contained 15% silver chloride in silver paste (MCA Services). The counter electrode and basal tracks were fabricated from 145R carbon ink (MCA Services). The basal tracks were insulated from the measurement solution using 242-SB epoxy-based protective coating ink (Agmet ESL, Reading, UK). The electrodes were then heat treated at 125 °C for 2 h, in order to cure the epoxy resin and to stabilise the electrocatalytic pad to allow prolonged use of the device in aqueous solutions.

Aliquots (10 μl) containing various amounts of ME in 10 mM buffer were pipetted on to the working electrodes, dried for > 40 min at room temperature and stored wrapped in silver foil at 4 °C until required.

Electrochemical test procedure

Measurements were performed using an Autolab Bipotentiostat Electrochemical Analyser with GPES3 software (Ecochemie, Utrecht, The Netherlands). A 1.1 cm diameter Whatman 114 filter disc (Whatman, Maidstone, UK) was placed over the three-electrode assembly, which, when wetted with sample, completed the electrochemical circuit. Buffer electrolyte (40 μl) containing 10 mM of NADP, was deposited on the filter-paper and the working electrode was poised at a potential of +300 mV *vs.* the Ag/AgCl reference. The amperometric measurement procedure was initiated and the electrochemical response was allowed to equilibrate for 240 s, after which 20 μl of sample solution were added to the filter-paper. The change in response was recorded at 350 s (*i.e.*, 110 s after sample addition). All test solutions were prepared in buffer–electrolyte and all tests were performed at 34 °C in accordance with the findings of Messia *et al.*[16] Since real samples contain appreciable levels of naturally electroactive species such as organic acids, the samples were tested simultaneously on the electrochemical device in the absence of immobilised enzyme, using the Autolab in bipotentiostat mode. The difference in current between the two responses was taken as a measure of specific malic enzyme activity and provided a simple means of accounting for interference factors. All tests were performed in triplicate.

Test kit method

Sensor performance was compared against a standard L-malic acid test method based on a commercially available colourimetric malic enzyme test kit (Dyzyme, Oxford, UK). Samples were tested in accordance with the supplied protocol and the absorbance was measured at 340 nm.

Determination of optimum detection potential for NADPH

The optimum detection potential for the oxidation of NADPH was determined by step-amperometry across the potential range −600 to +600 mV using potential and time increments of 100 mV and 200 s, respectively. Tests were performed on blank electrodes in glass beakers containing 10 ml of stirred buffer–electrolyte. Current responses were recorded immediately before each potential step in the presence or absence of 10 mM NADPH.

Tests on real samples

Samples were prepared using a Tefal multi-purpose grater (Product code 6303, Lakeland, Cumbria, UK) and operated according to the manufacturer's instructions. In outline, a manually driven screw is used to push diced sample through the grater. Therefore, the pressure applied to the system is primarily a function of the inherent resistance provided by the sample at it is forced through the grater. Juice was extracted from grated samples by wrapping 100 g of material in fine food-grade cotton mesh (plain Voile, product code K656 40921, John Lewis, Milton Keynes, UK). and applying sufficient pressure to recover produce juice. The mesh acted as a crude filter to minimise the presence of large particulates in the juice extracts. Samples were diluted in buffer as follows: Jonagold apple, ×100; Bramley apple, ×200; Russell Burbank potato, ×10; and mini plum tomato, ×10; they were then tested simultaneously using the biosensor and test kit method. The extraction method chosen was designed to be simple, rapid and easy to apply in the field. Three different potato and tomato samples and six different apple samples were analysed in triplicate.

Results and discussion

Selection of optimum measurement conditions

A pH of 7.4 and a temperature of 34 °C were used in all tests based on previous biosensor studies using ME.[15,16] The active site of malic enzyme is thought to be a sulfhydryl group, which can be stabilised using 20 μM 2-mercaptoethanol (2-MCE).[17] ME also requires trace amounts of divalent cations such as Mg^{2+} or Mn^{2+}. The ME stock solution used in this study contained 0.5 mM 2-MCE and 10 mM $MnCl_2$, which proved adequate for sensor performance even after buffer dilution (addition of 20

μM 2-MCE and 3 mM $MnCl_2$ to the buffer–electrolyte had no measurable effect upon sensor performance).

Choice of working electrode material and detection potential

The electrochemical behaviour of NADPH and a range of potential interferents with detection potential using both bare carbon and rhodinised carbon is shown in Fig. 1. In all cases, increases in potential resulted in increased current responses. The highest responses were observed for L-ascorbic acid on both electrode types. However, rhodinised carbon proved superior for the oxidation of NADPH at applied potentials of +200 mV or greater compared with bare carbon. Using zero current as the baseline, the response ratios for L-ascorbic acid:NADPH:glutamic acid (the most responsive of the other organic acids tested) at potentials of +200 and +300 mV were 10.6:6.6:1.0 and 4.3:4.3:1.0, respectively on rhodinised carbon. On bare carbon, corresponding response ratios of 85.2:2.4:1.0 and 76.4:3.3:1.0 were recorded at the same respective potentials.

Although the rhodinised carbon gave higher response values, the signal-to-noise ratios (S/N) obtained were similar to those obtained for the bare carbon electrodes. For both carbon types, the highest S/N values recorded (~ 120:1) for NADPH were at +300 mV. A maximum S/N value was apparent at the same potential for ascorbic acid, although the other acids showed an increase in S/N at the higher potentials. The data suggest that there is no benefit in measuring at higher potentials since no improvement in the NADPH response relative to the selected interferents is apparent. Higher detection potentials increase the likelihood of the oxidation of other interferents. Rhodinised carbon, with a detection potential of +300 mV, was used in subsequent studies.

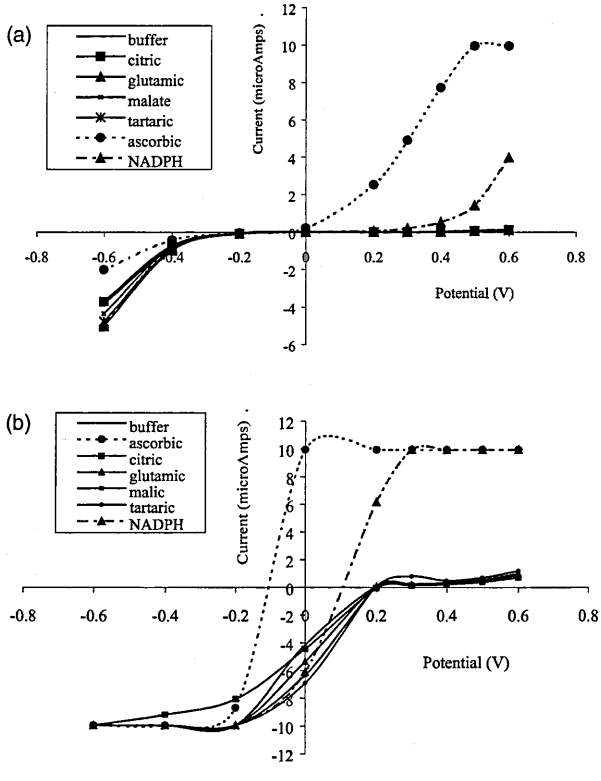

Fig. 1 Current *versus* potential profile for NADPH co-factor and a range of possible organic acid interferents measured on (a) bare carbon and (b) MCA4a rhodinised carbon. 10 mM preparations were used with the mean of triplicate tests shown.

Ascorbic acid oxidase could be used to convert the highly electroactive ascorbic acid into dihydroxoascorbic acid and water but would represent an additional preparation step with the removal of only one interferent species. In this study, the approach used was to subtract the background response of each sample using electrodes without enzyme from the equivalent enzyme electrode response. A final device would therefore incorporate two working electrodes—both rhodinised carbon, but only one dosed with enzyme—and a common reference and counter electrode. The difference between the enzyme electrode and 'compensator' electrode responses would represent the L-malic acid-specific response.

Optimisation of enzyme loading activity

Initial tests were focused on determining the minimum enzyme activity required to generate a maximum current response from the system. In order to ensure that substrate concentration was not a limiting factor in the biosensor response, tests were performed under saturating levels of L-malic acid. A 2 mM L-malic acid solution was used, based on the criterion of saturation of $5 \times K_m$, where $K_m = 3.9 \times 10^{-4}$ M for malic enzyme from liver using L-malic acid as substrate.[23]

The amperometric response of sensors containing 0–0.6 mU malic enzyme per electrode to 2 mM L-malic acid in buffer–electrolyte were determined, with a maximum sensor response recorded at enzymes loading >0.35 mU per electrode. Accordingly, enzyme loadings of 0.38 mU per electrode were subsequently used to ensure an enzyme excess to maintain maximum biosensor response using a minimum amount of enzyme.

Sensor analytical performance

A linear relationship between L-malic acid concentration (x) and current response (y) was observed up to 0.7 mM [$y = 9.24 \times 10^{-7}x + 5.77 \times 10^{-8}$; correlation coefficient (r^2) value, 99.67%; analysis of variance (ANOVA) F significance value, 1.17×10^{-8}]. The limit of detection (LOD) for L-malic acid, calculated as 2.5× the standard deviation of the zero analyte response, was 0.028 mM. Hence the linear dynamic range of the system was 0.028–0.7 mM. The relative standard deviation (RSD) values varied from 3.3 to 13.3% (six concentrations, n = 3). Across the range 0–1 mM L-malic acid, the data were best described by a polynomial relationship, $y = 6 \times 10^{-7}x^4 - 2 \times 10^{-6}x^3 + 2 \times 10^{-6}x^2 + 6 \times 10^{-7}x + 7 \times 10^{-8}$; $r^2 = 0.9990$.

The sensor was also compared against a commercially available standard colorimetric malic acid test kit (Fig. 2). A

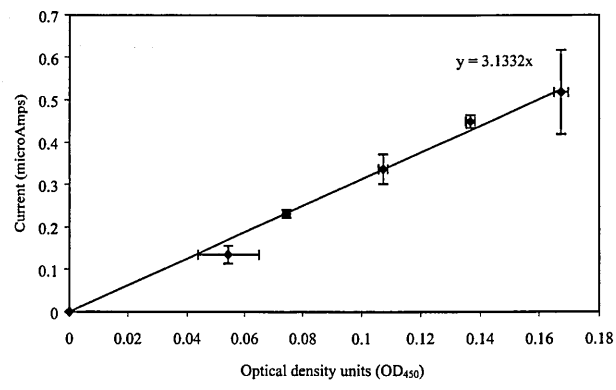

Fig. 2 Correlation between electrochemically and photometrically determined L-malic acid concentrations. Background response values have been subtracted. Error bars = s, n = 3.

simple linear relationship ($y = 3.1332x$; $r^2 = 99.23\%$; F significance value, 2.25×10^{-5}) was observed. The concentration range over which both methods can be directly compared was 0.028–0.7 mM, dictated by the linear range of the electrochemical method.

Inhibition effects

In addition to the electrochemical interferent effect [Fig. 1(b)], a number of compounds found in horticultural samples are also known to inhibit ME activity by competing with L-malic acid for enzyme binding sites. Since notable inhibitors include L-aspartic acid and L-citric acid, the same four organic acids as examined in the interference studies were evaluated with regard to their inhibitory effect on ME enzyme.

First, it was necessary to determine the extent of the sensor signal due to interference effects. The current response due to the direct electro-oxidation of 0.3 mM of the selected organic acids was determined on enzyme-free rhodinised carbon electrodes. The results are given in Table 1, column 2. As indicated previously [Fig. 1(b)], L-ascorbic acid gave the highest response (197 nA) with all other acids giving responses <75 nA.

Next, the response of the enzyme electrode to each organic acid interferent was determined. No significant changes in sensor response were noted, indicating no reaction between the enzyme and these compounds (Table 1, column 3). Inhibitory effects were then determined by preparing solutions containing 0.3 mM L-malic acid and 0.3 mM organic acid interferent and determining the biosensor response (column 4). In all cases, an increase in sensor response was obtained owing to the presence of L-malic acid substrate. The difference between the enzyme electrode and enzyme-free electrode response equated to the sensor response due to L-malic acid oxidation (column 5). Comparing the size of these responses to the L-malic acid response indicated no significant change in the enzyme electrode performance (column 6). Therfore, it was concluded that inhibition of ME by other organic acids was not significant using the enzyme electrode.

Testing real samples

A study by Hulme and Rhodes[23] indicated that L-malic acid constitutes 90% of the total organic acid content in apples. Akermann *et al.*[24] showed that an elevated amount of malic acid is present in immature fruits, including apples, which decreases as the fruit ripens. Measurement of L-malic acid therefore has possibilities as an objective indicator of fruit maturity. Correspondingly, the performance of the sensor in measuring L-malic acid levels in apple, potato and tomato preparations was determined and the results were compared against the standard photometric test kit. The selected sample preparation method was chosen for ease of performance in the field. All samples were prepared freshly and tested simultaneously using the two methods. The results are given in Table 2. Russell Burbank potatoes, mini plum tomatoes and Jonagold and Bramley apple varieties were found to have L-malic acid concentrations of 3–4, 2.0–2.7, 30–60 and 80–120 mM, respectively.

The accuracy of the enzyme electrode response was determined against the test kit method using the equation: accuracy $= [(S_R - K_R)/K_R] \times 100\%$ where S_R is sensor response and K_R is test kit response. In all cases, the sensor response was within 13.7% of the response of the standard method. Although improved accuracy would be desirable, the sensor method does provide a simple and field-based method for indicating the levels of L-malic acid in the tested horticultural samples, thus

Table 1 L-Malic acid biosensor inhibition studies. Full details of the method used to calculate malic enzyme inhibition by the selected organic acids are provided in the text. Values are shown ±1s (n = 3)

Interferent/inhibitor	Rhodinised carbon electrode response/nA[a]	Enzyme electrode response/nA[a]	Enzyme electrode response to malic acid + interferent/nA	Malic acid-specific response/nA[c]	Percentage of malic acid-specific response[d]
L-Malic acid	68 ± 1.9	337 ± 35.1	350 ± 61.8[a]	282	100.0
L-Tartaric acid	45 ± 4.2	62 ± 19.1	334 ± 39.4[b]	289	102.6
L-Glutamic acid	75 ± 15.2	53 ± 14.3	355 ± 71.2[b]	280	99.2
L-Citric acid	73 ± 13.0	68 ± 36.2	357 ± 42.8[b]	284	100.5
L-Ascorbic acid	197 ± 57.6	206 ± 6.6	474 ± 119[b]	277	98.2

[a] 0.3 mM of analyte. [b] 0.3 mM of analyte + 0.3 mM of L-malic acid. [c] Difference between columns 2 and 4. [d] Calculated as (column 5 response value/column 5 response value for L-malic acid) × 100%.

Table 2 Comparison of sensor and standard photometric test kit results for measurement of L-malic acid in potato and apple samples. Values are shown ±1s (n = 3)

Sample	Dilution	Sensor response, S_R/mM	Test kit response, K_R/mM	Accuracy (%)[a]
Potato (Russell Burbank)	×10	0.353 ± 0.055	0.337 ± 0.019	4.8
Potato (Russell Burbank)	×10	0.380 ± 0.010	0.335 ± 0.096	13.4
Potato (Russell Burbank)	×10	0.309 ± 0.008	0.334 ± 0.09	−7.5
Apple (Jonagold)	×100	0.552 ± 0.088	0.488 ± 0.086	13.1
Apple (Jonagold)	×100	0.479 ± 0.059	0.542 ± 0.019	6.0
Apple (Jonagold)	×100	0.378 ± 0.084	0.388 ± 0.081	−2.6
Apple (Bramley)	×200	0.391 ± 0.008	0.419 ± 0.036	−6.7
Apple (Bramley)	×200	0.465 ± 0.095	0.429 ± 0.012	8.4
Apple (Bramley)	×200	0.582 ± 0.208	0.579 ± 0.124	0.5
Tomato (mini plum)	×10	0.234 ± 0.124	0.269 ± 0.020	−13.0
Tomato (mini plum)	×10	0.201 ± 0.035	0.233 ± 0.010	−13.7
Tomato (mini plum)	×10	0.270 ± 0.047	0.258 ± 0.010	4.7

[a] Accuracy $= [(S_R - K_R)/K_R] \times 100\%$, where S_R is the sensor response and K_R is the test kit response.

providing a more objective assessment of produce maturity and taste. The sensor method offers some advantages over the test kit method, particularly with regard to field-based measurements since the sensor merely requires the dilution and addition of prepared sample to the filter disc, whereas the test kit method requires a number of sample and reagent handling steps. Less training is required to operate the sensor method, which is more rapid (6 *vs.* 24 min) with lower labour, disposables and reagent costs. However, the linear range of the sensor is 0.028–0.7 mM compared with 0.0038–2.5 mM for the test kit.

In addition to horticultural produce assessment, there may also be other potential applications for the L-malic acid biosensor. According to Palleschi,[25] the quality of red and white wines and their organoleptic characteristics are very much dependent upon the extent of internal malo-lactic fermentation. The net result of this type of fermentation is the formation of lactic and malic acids, which will influence the taste of the wine product. Palleschi further stated that the biosensor approach matches the requirements of wine producers with regard to selective and rapid determination of lactate and malic acid in wine.

The principle organic acid in apple juice is L-malic acid (0.15–0.91% w/w) and no D-malic acid should be present. Since L-malic acid is expensive, it is not economic for use as a product adulterant. However, synthetic D,L-malic acid is cheap to produce. In unadulterated apple juice, the L-malic acid to total malic acid ratio is 1.0, compared with 0.5–1.0 for adulterated juice. The current way of assessing apple juice adulteration is to measure total malic acid by HPLC and L-malic acid using enzyme-based test kits.[26] The L-malic acid biosensor could have applications in place of the test kit where a limited number of simple, rapid measurements are preferred.

Conclusions

Screen-printed electrodes based on malic enzyme with amperometric measurement of NADPH oxidation have proved suitable for the simple, low-cost and rapid determination of L-malic acid in apple, potato and tomato samples. Addition of mediator is not required since the rhodinised carbon working electrode favoured the oxidation of NADPH at a lower operating potential (+300 mV *vs.* Ag/AgCl) compared with a number of possible organic acid interferents, except L-ascorbic acid. Residual interference effects can be accounted for through the use of a compensator electrode. The sensor performance was tested against a photometric kit using real samples, yielding accuracy values within 13.7% of the standard method. Since the measurement process is simple, it is amenable to field-based usage with a minimal training requirement. Low sensor manufacturing costs result in single-use disposable devices, thus negating problems of progressive electrode fouling.

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